THE EFFECT OF ALTERATIONS OF THE COMPONENTS OF THE BICARBONATE BUFFER SYSTEM ON SKELETAL MUSCLE CONTRACTION, POTASSIUM CONTENT AND RESTING POTENTIAL

By
David W. Fretthold

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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David W. Fretthold

Chairperson: Dr. Lal C. Garg
Department: Pharmacology and Therapeutics

The purpose of this investigation was to examine the effect of alterations in bicarbonate concentration and carbon dioxide tension on skeletal muscle contractility and to elucidate a mechanism for these effects. Hemidiaphragm muscles from control rats and rats fed potassium-deficient chow were examined. Particular attention was focused on this potassium-depleted muscle because it has been reported to exhibit some of the same properties as muscle in patients suffering from the hereditary disease hypokalemic periodic paralysis. Patients with this disease are protected from attacks of skeletal muscle paralysis by chronic doses of ammonium chloride or carbonic anhydrase inhibitors, agents that produce alterations in the bicarbonate buffer system.

The *in vitro* situation offers the opportunity to control carefully all three elements of the buffer system: pH, bicarbonate, and carbon dioxide tension. Therefore, all muscle

studies were conducted *in vitro*. One of these three elements was kept constant while the other two were systematically varied.

When pH was allowed to vary, stimulated muscles contracted with more force in high pH buffers than in low pH buffers. This was true when the pH change was effected by an elevated buffer bicarbonate concentration or by a reduced carbon dioxide tension. This effect was always more dramatic in muscles taken from potassium-depleted animals. With these muscles, a change in pH from 7.7 to 7.1 was accompanied by a 65% reduction in contractile force. This effect showed a rapid onset, was reversible, persisted until the buffer composition was changed, and was independent of synaptic transmission. When pH was kept constant but buffer bicarbonate and carbon dioxide tension were increased together at the same ratio, there was an accompanying increase in muscle contractile force. This relationship was also more pronounced in potassium-deficient rat muscles.

Intracellular pH and intracellular potassium determinations were performed on diaphragm muscles that had been contracting in different bicarbonate buffers. Radio-labeled dimethyloxazolidinedione (DMO) was used for these intracellular pH determinations. Muscles bathed in acidic buffers were found to have lower intracellular pH levels and lower intracellular potassium concentrations than muscles bathed in more alkaline buffers. Analysis of muscles bathed in high bicarbonate, high carbon dioxide buffer revealed that these muscles had a lower intracellular pH and higher

potassium content than muscles bathed in low bicarbonate, low carbon dioxide buffer, although both buffers had the same pH.

Resting membrane potentials, recorded in diaphragm muscles bathed in different bicarbonate buffers, were not affected by changes in buffer bicarbonate concentration or buffer carbon dioxide tension. Changes in the buffer potassium concentration over ranges which did not affect contractile response were found to alter the recorded resting potentials.

The changes in contractility showed a positive correlation with the intracellular muscle potassium concentration and with the ratio of intracellular to extracellular proton concentrations. Changes in muscle contractility did not correlate with changes in intramuscular pH or with changes in the resting membrane potential. It was speculated that the benefits which carbonic anhydrase inhibitors afford victims of hypokalemic periodic paralysis may be derived from a more favorable distribution of potassium which results from reductions in serum bicarbonate as well as the accompanying acidosis.

INTRODUCTION

Objective

Patients suffering from the disease familial hypokalemic periodic paralysis are protected from attacks of muscle weakness by acetazolamide, a potent carbonic anhydrase inhibitor (Resnick et al., 1968). It is paradoxical that a diuretic which causes potassium loss can prevent the attacks of muscle weakness which are associated with a fall in serum potassium. Since no carbonic anhydrase has been found in muscle (Maren, 1967), it seems unlikely that the benefits afforded victims of this malady are derived from action of the drug directly on muscle. Griggs (1970) suggested that metabolic acidosis, brought about by inhibition of renal carbonic anhydrase, was responsible for the beneficial effects of this drug. Jarrell et al. (1976) demonstrated that patients given acidifying doses of ammonium chloride also were protected from periodic attacks. Further, Viskoper et al. (1973) were able to precipitate an attack of paralysis in a patient by infusing sodium bicarbonate which produced a metabolic alkalosis. Treatment with acetazolamide appears to prevent hypokalemia and muscle paralysis as a consequence of alterations effected in the bicarbonate buffering system. These alterations include not only a decrease in the plasma pH, but also decreases in plasma bicarbonate and carbon

dioxide concentrations. Precisely how this new acid-base equilibrium prevents attacks of periodic paralysis is not clear. Therefore, the objective of this investigation is to determine *in vitro* the influence of changes in components of the bicarbonate buffer system on skeletal muscle contraction, potassium content, pH, and resting membrane potential.

Background

Bicarbonate Buffer System

The bicarbonate buffer system is quantitatively the most important extracellular buffer in the body and is unique because of its intimate link with carbon dioxide. Although the ionization constant (pKa) for the first proton dissociation of carbonic acid is 3.6, the buffer system has a considerable capacity at physiological pH because of the constant 400:1 ratio of dissolved carbon dioxide to carbonic acid. The Henderson-Hasselbalch equation can be modified, substituting the concentration of dissolved carbon dioxide divided by 400 for the carbonic acid concentration. Rearranging the terms yields the familiar form of the equation which expresses the pH as a function of bicarbonate concentration and dissolved carbon dioxide, with an apparent pKa value of 6.1 (Hills, 1973); $pH=6.1+\log\frac{(HCO_3^-)}{(CO_2)}$. *In vivo*, regulatory mechanisms control the ventilation rate and consequently the carbon dioxide concentration in blood, thus controlling the

concentration of the proton donor, carbonic acid. Extracellular pH is maintained between 7.1 and 7.6, the range generally considered compatible with life. Even during strenuous exercise, when the carbon dioxide evolution rate can increase to as much as thirty times the rate at rest, extracellular pH falls no more than 0.1 unit.

Relation Between Intracellular and Extracellular pH and Bicarbonate of Muscle Cells

A variety of techniques have been employed to determine the intracellular pH of muscle. Early methods used to measure intracellular pH and the theoretical and practical difficulties associated with these methods are discussed in a review by Caldwell (1956). Waddell and Butler (1959) first used the weak acid, dimethyloxazolidinedione (DMO), a metabolite of the antiepileptic agent trimethadione, to measure the intracellular pH of dog muscles *in vivo*. Using bicarbonate-specific microelectrodes, Khuri et al. (1974, 1976) determined the intracellular bicarbonate activity of skeletal muscles, and calculated equilibrium potentials for bicarbonate of between -20 millivolts and -44 millivolts under different conditions in rat skeletal muscles. These authors and others have determined intracellular pH to be between 6.6 and 7.4, and have concluded that proton and bicarbonate gradients must exist across muscle cell membranes which are not in Nernstian equilibrium with the resting muscle membrane potential, although the membrane is permeable to these ions (Adler et al., 1965). In order for such gradients to be maintained, either hydrogen ions would have to

be transported out of cells or bicarbonate ions would have to be transported into cells (Adler et al., 1972; Heisler, 1975; Khuri et al., 1974; Lai et al., 1973).

Numerous investigations have been undertaken to determine the relation of intracellular pH to changes in extracellular pH. This was the objective of Waddell and Butler (1959) in their DMO study in dogs. They altered the blood carbon dioxide tension and bicarbonate concentration and concluded, from skeletal muscle biopsies, that the greatest changes they observed in intracellular pH resulted from changes in carbon dioxide tension. Irvine and Dow (1966) found that metabolic acidosis in rats had no effect on skeletal muscle intracellular pH. Kim and Brown (1968) showed that skeletal muscle intracellular pH in dogs fell when carbon dioxide tension was increased along with bicarbonate concentration, and rose when animals were hyperventilated during HCl infusion, even though the plasma pH remained constant. Similar results have been obtained in vitro; respiratory acidosis produced larger alterations in muscle intracellular pH than metabolic acidosis (Heisler, 1975; Adler et al., 1965). Both these authors found that rat diaphragm muscles were most effectively buffered when extracellular pH ranged between 7.1 and 7.4. They reported that when extracellular pH varied from 7.1 to 7.4 during simulated respiratory acidosis or alkalosis, intracellular pH, measured with DMO, varied from 7.0 to about 7.28. Outside this extracellular pH range, intracellular pH changed about as much as extracellular pH.

Electrolyte Shifts and Acid-Base Disturbances

A close relationship exists between tissue and plasma electrolyte concentrations and acid-base balance. Potassium depletion results in increases in serum bicarbonate (Heppel, 1939), whereas sodium depletion leads to a fall in serum bicarbonate (Darrow et al., 1948). Acidosis produces elevations in total body potassium, and decreases in total body sodium (Cooke et al., 1952). All of the above studies were chronic experiments involving chronic alterations in diet and acid-base balance and the changes largely result from renal electrolyte regulation. A reciprocal relation exists for hydrogen and potassium ion excretion in the distal tubule, and reabsorption of bicarbonate in the proximal tubule is coupled to the reabsorption of sodium (Pitts, 1963).

In a series of experiments on nephrectomized dogs, Pitts and co-workers (1963) measured serum ion concentrations and demonstrated that considerable ion exchange occurs between intracellular and extracellular fluid in acute episodes of respiratory and metabolic acidosis and alkalosis. Sodium and potassium exchange for protons, and chloride exchanges for bicarbonate. Other authors have analyzed muscle taken from animals during acid-base disturbances and have noted positive correlations between hydrogen and potassium ion gradients across muscle membranes (Brown and Goott, 1963; Grantham and Schloerb, 1965; Irvine and Dow, 1966). In various types of acid-base alterations, intramuscular potassium concentration changed in the direction which tended to keep the transmembrane ratios of potassium

and hydrogen ions equal, although true equality was never attained (Brown and Goott, 1963). In vitro experiments on rat diaphragms by Adler et al. (1965) and on frog muscles by Fenn and Cobb (1934) have shown a positive correlation between changes in intramuscular potassium and intramuscular pH. In vitro potassium depletion in rat diaphragm has been shown to lower intracellular pH (Adler et al., 1972). Skeletal muscles depleted of potassium contain elevated concentrations of sodium (Cooke et al., 1952).

Mechanism of Ion Exchange.

Isotope exchange studies with isolated frog skeletal muscle have shown that a decrease in extracellular pH causes an increase in the potassium efflux rate, a decrease in the potassium uptake rate, and a bi-directional depression of sodium exchange (Volle, 1972). This type of membrane behavior could explain the potassium shift seen in acidosis (Volle, 1972). Keynes and Swan (1959) reported that sodium efflux from isolated frog muscle varied approximately with the cube of the internal sodium concentration. Keynes (1965) found that sodium efflux depended on the square of the internal sodium concentration in acidosis which was produced by an increase in carbon dioxide tension. Whether these observations represent interactions of ion pumps, substitution of protons for other cations, or suppression of pumping activity is not known.

Muscle Cell Potential and pH, Bicarbonate Concentration, and Carbon Dioxide Tension

Resting muscle membrane potential depends on the ion distribution across the membrane and the membrane permeability to these ions. The Goldman equation describes this relationship quantitatively:

$$E_m = \frac{-RT}{F} \ln \frac{P_K(K^+)_i + P_{Na}(Na^+)_i + P_{Cl}(Cl^-)_o}{P_K(K^+)_o + P_{Na}(Na^+)_o + P_{Cl}(Cl^-)_i}$$

where E_m is membrane potential, P is the membrane permeability coefficient for a particular ion, R is the universal gas constant, T is temperature in degrees kelvin, and F is Faraday's constant (Hodgkin and Horowicz, 1959). In skeletal muscle, chloride is distributed in agreement with E_m, and the contribution of the term $P_{Na}(Na^+)_i$ is small compared to the contribution of the term $P_K(K^+)_i$. Therefore, these terms may be neglected without appreciably altering the validity of the equation (Bilbrey et al., 1973). Bilbrey et al. (1973) found that resting muscle potentials, recorded in vivo in dogs and rats, agreed with this equation when 0.01 was used for the ratio of the permeability coefficients of sodium to potassium. Any factor which influences a change in any of the parameters in the equation would affect the membrane potential. Studies on the effect of pH and carbon dioxide tension on transmembrane potential have yielded conflicting results. In general, an increase in carbon dioxide tension has been shown to cause a decrease in E_m when E_m is initially high and an increase when E_m is initially low (Shanes, 1958).

In vivo studies by Williams et al. (1971) showed that a fall in blood pH induced by an elevation in pCO_2 causes a slow depolarization of skeletal muscle in rat. This depolarization could be explained by the observed redistribution of ions, principally an elevation in plasma potassium. *In vitro* alterations in pH have been shown to affect membrane potential in frog muscle; the effect has been attributed to a decreased chloride permeability in acidosis (Brooks and Hutter, 1962; Hutter and Warner, 1967); when the external potassium concentration was 2.5 mM, acidosis hyperpolarized isolated frog skeletal muscle by a few millivolts, but when the external potassium concentrations was elevated to 10 mM, acidosis depolarized the muscle by a few millivolts (Mainwood and Lee, 1967). It has also been reported in rat skeletal muscle that bicarbonate has a permeability coefficient approximately 0.1 that of chloride (Hugeunin, 1975).

Muscle Contraction

The first in a series of events leading to muscle contraction is usually the propagation of an action potential to the pre-synaptic ending of the motor nerve, and the subsequent release of acetylcholine from the nerve ending. Acetylcholine diffuses across the synaptic cleft and interacts with the post-synaptic membrane on the muscle, causing a temporary local increase in permeability to sodium and potassium. As a result of this permeability increase, the

membrane becomes locally depolarized. If the muscle membrane depolarizes beyond a critical threshold, a propagated action potential results. Depolarization of the muscle membrane initiates the liberation of calcium from sarcoplasmic reticulum, the primary source of activator calcium in skeletal muscle. Movement of calcium to the contractile apparatus of the muscle links the excitation process to the contractile process. At low intramuscular calcium concentrations (less than 10^{-6} M), the muscle fiber protein, actin, is bound to the regulator proteins, tropomyosin and troponin, and actin remains in an inactive state. After calcium release, calcium binds to troponin, and actin complexes with myosin, the other primary contractile protein. Several subsequent steps result in the hydrolysis of ATP by myosin ATPase and the shortening of the sarcomere. Calcium is then actively pumped back into the sarcoplasmic reticulum, and the formation of actin-myosin contractile complexes is inhibited and contraction ceases. For a more complete treatment of this subject, see Tonomura (1973) and Weber and Murray (1973).

Effect of Acidosis on Muscle Contraction

As an interesting corollary of studies on the effect of pH on the neuromuscular blocking agents, Baraka (1964) and Crul-Sluijter and Crul (1974) observed a slight depressive effect of acidosis alone on skeletal muscle twitch response. Baraka (1964) conducted his experiments on humans *in vivo* and interpretation of his results is complicated because acidosis also effects elevations in serum sodium and potassium

concentrations and in levels of circulating epinephrine and norepinephrine (Sechzer et al., 1960). However, experiments by Crul-Sluijter and Crul (1974) with in vitro rat diaphragm preparations show that muscles contract with slightly less force when the bath fluid is made acidic by decreasing the bicarbonate concentration. The mechanism of this effect has not been determined, and is is not clear whether the depression is dependent on the change in pH or the change in the bicarbonate concentration.

Potassium Deficiency with Reference to Skeletal Muscle

Potassium deficiency is manifested differently in different species. In both dog and rat, the decrease in intramuscular potassium concentration is relatively less than the decrease in extracellular potassium concentration (Bilbrey et al., 1973). In man, serum potassium concentrations may remain near normal despite severe potassium loss from muscles (Rettori et al., 1972). Potassium deficiency in rats causes characteristic myocardial and renal damage (Welt et al., 1960), but no skeletal muscle paralysis develops, even in severe potassium deficiency (Smith et al., 1950). Resting muscle membrane potentials in potassium-deficient rats were found to be in agreement with values predicted by the Goldman equation (Bilbrey et al., 1973). In both dog and man, progressive skeletal muscle weakness and structural degeneration develop (Chanpion et al., 1972; Smith et al., 1950). Bilbrey et al. (1973) have attributed muscle weakness in severely potassium-deficient dogs to an abnormally low resting membrane potential. They

suggested that these low resting potentials resulted because the membrane permeability to sodium relative to the permeability to potassium had increased, although they did not test this hypothesis. Potassium release from exercised muscle, which is thought to mediate increased local blood flow, has been demonstrated to be severely reduced in potassium-deficient dog (Knochel and Schlein, 1972). Muscle necrosis and associated myoglobinuria following exercise in potassium-depleted dog and man have been attributed to failure of these muscles to show the normal hyperemic response to exercise.

Muscle Paralysis and Potassium Distribution

There are several conditions in which transitory muscular paralysis is associated with abnormal potassium distribution. Two such kinds of paralysis seen in humans are hyperkalemic familial periodic paralysis, also known as adynamia episodica hereditaria, and hypokalemic periodic paralysis. In hyperkalemic periodic paralysis, attacks of muscle weakness are associated with elevations in serum potassium (Gamstorp et al., 1957). Acetazolamide and hydrochlorothiazide are used to control this disease, presumably acting by lowering the serum potassium through kaluresis (McArdle, 1969).

Acetazolamide, a potent carbonic anhydrase inhibitor and potassium wasting diuretic, has been found to be the most effective treatment available for hypokalemic periodic paralysis (Griggs et al., 1970). In hypokalemic periodic paralysis, attacks are associated with a fall in the serum potassium concentration (Biemond and Daniels, 1934). During attacks,

potassium has been shown to move from serum into skeletal muscle (Ionasescu and Merculiev, 1962), and serum potassium concentrations fall to 2.5 mM or lower (Vroom et al., 1975). During attacks there is a depression of muscle electrical activity (Viskoper et al., 1973) and muscles have been reported to be depolarized rather than hyperpolarized as would be expected on the basis of the abnormal potassium distribution (Shy et al., 1961; Creutzfeldt et al., 1963). These observations are consistent with the theory that muscles are paralyzed by a depolarization block resulting because of a decrease in the potassium permeability of the muscle membrane. The sodium contribution to the resting potential becomes relatively more important, and depolarization results. During depolarization, the muscle membrane sodium-potassium ATPase activity increases and the serum potassium concentration continues to fall (Otsuka and Ohtsuki, 1970). Although increasing the extracellular fluid potassium concentration might be expected to further depolarize the muscle membranes, potassium chloride infusion terminates the paralysis (Aitken et al., 1937).

Diaphragm muscles isolated from potassium-deficient rats have several characteristics in common with muscle in hypokalemic periodic paralysis patients, although these rats do not suffer paralysis in vivo (Smith et al., 1950). Muscles in periodic paralysis patients and in potassium-deficient rats contain elevated intracellular sodium and reduced intracellular potassium (Hoffmann and Smith, 1970; Offerijns et al., 1958). Offerijns et al. (1958) reported that insulin caused flacid paralysis in the hemidiaphragm preparation in

conjunction with low potassium in the buffering medium. Insulin, which lowers serum potassium more in hypokalemic periodic paralysis patients than in normal persons, also induces severe attacks of paralysis in these patients (Vroom et al., 1975), although it is not clear whether insulin is essential in all episodes. In potassium-deficient rat diaphragm muscle paralysis, as in hypokalemic periodic paralysis, the resting muscles have been reported to be depolarized while the extracellular potassium concentration remains reduced; paradoxically normal contractile function could be restored in both cases by elevating the extracellular potassium concentration (Gordon et al., 1970; Ohtsuka and Ohtsuki, 1970).

RATIONALE

Changes in pH, with accompanying changes in either carbon dioxide tension or bicarbonate concentration, affect several important skeletal muscle properties. These properties include ion distribution, cell permeability, cell potential, and contractile force. Each of these factors plays a vital role in muscle performance, but the influence of the bicarbonate buffer system on these properties has never been extensively investigated. Therefore, we measured skeletal muscle contractile force, cation composition, and resting membrane potential in _in vitro_ experiments using three different bicarbonate buffer series. One of these series had buffers with different carbon dioxide concentrations, simulating the changes seen in respiratory acid-base disturbances. One series had buffers with different bicarbonate concentrations, simulating metabolic acid-base disturbances. The third series had buffers with different concentrations of bicarbonate equilibrated with different carbon dioxide mixtures, but with the same ratio of $HCO_3^-:CO_2$, and hence, with the same pH. Through this approach we hoped to determine whether bicarbonate and carbon dioxide have any specific effects on skeletal muscle apart from changes in pH.

Rat diaphragm muscle from both normal and potassium-depleted rats was studied in these experiments. Potassium-

deficient rat diaphragm muscle was selected for investigation since it previously has been shown to be similar in many respects to skeletal muscle of hypokalemic periodic paralysis patients. Further, the rat diaphragm muscle is less than on millimeter thick, and can easily be oxygenated in vitro. The main objective was to determine whether potassium-depleted rat muscle shares with hypokalemic periodic paralysis muscle a sensitivity to changes in the bicarbonate buffer system. If such a bicarbonate-carbon dioxide sensitivity were found, perhaps an investigation of the mechanism would provide a better understanding of the influence of acid-base balance upon skeletal muscle and aid in elucidation of the effect of carbonic anhydrase inhibitors upon hypokalemic periodic paralysis.

SPECIFIC AIMS

The specific aims of this investigation are to determine in both normal and potassium-depleted rat muscle:

1. the effect of alterations in bicarbonate concentration, carbon dioxide tension and pH on contractility
2. the effect of alterations in bicarbonate concentration, carbon dioxide tension, and pH on muscle potassium content
3. the effect of alterations in bicarbonate concentration, carbon dioxide tension, and pH on muscle intracellular pH
4. the effect of alterations in bicarbonate concentration, resting carbon dioxide tension, and pH on membrane potential
5. the effect of variations in the extracellular potassium concentration on resting muscle membrane potential

MATERIALS AND METHODS

Buffer Composition

Muscle experiments were performed in Tyrode solution of the following composition: NaCl, 126 mM; KCl, 5 mM; $CaCl_2$, 3 mM; $MgCl_2$, 1.6 mM; dextrose, 11.1 mM; Na_2HPO_4, 0.677 mM; NaH_2PO_4, 0.172 mM; and $NaHCO_3$, 16 mM. Low bicarbonate solutions were made by substituting 8 millimoles per liter of sodium chloride for 8 millimoles per liter of sodium bicarbonate. High bicarbonate solutions were made by substituting 16 millimoles per liter of sodium bicarbonate for 16 millimoles per liter of sodium chloride. In one series of experiments, the sodium chloride concentration was kept constant at 118 mM, and 0.5 millimoles sodium sulfate plus 0.5 millimoles sucrose were substituted for each millimole of sodium bicarbonate in reduced bicarbonate solutions. Low potassium solutions contained 0.5 mM potassium chloride plus an additional 4.5 millimoles per liter of sodium chloride. Elevated potassium solutions were prepared by substituting potassium chloride millimole for millimole for sodium chloride.

Solutions were gassed with mixtures of oxygen and carbon dioxide. The gas mixtures contained oxygen with either 2.5%, 5%, or 10% carbon dioxide by volume. The mixture with 2.5% carbon dioxide was obtained from and certified by Matheson

Products, and the mixtures with 5% and 10% carbon dioxide were obtained from Liquid Air Company.

Muscle experiments were performed in one of the three series of buffers illustrated in Table 1. The pH of the buffer solutions was measured near the tissue site in all experiments. The pH was measured with an Arthur Thomas Company combination electrode and a Beckman pH meter. It was necessary to carefully regulate the bubble rate of the gas, and to compensate for cylinder variations in gas mixtures by varying the bicarbonate concentrations slightly when dealing with the constant pH buffers (series three buffers). Bicarbonate concentrations other than those shown in Table 1 were used, although none of these values varied more than 25% from the values expressed in the table. The bicarbonate concentrations illustrated in Table 1 represent approximate concentrations as well as approximate carbon dioxide volumes percent of the gases.

Experimental Animals

Male Sprague-Dawley rats, 100-150 g, obtained from Flow Labs, were housed individually in wire cages. They were fed potassium-deficient rat chow, completely supplemented with vitamins and minerals, obtained from Nutritional Biochemicals. Rats were maintained on this diet for 5 to 6 weeks prior to the experiment. At the time of the experiment, these rats weighed between 200 g and 275 g. Male Sprague-Dawley rats, weighing 200-275 g, obtained from Flow Labs, were used as control rats. These animals were fed standard rat chow.

TABLE 1

BUFFER SERIES

	HCO_3^- / CO_2 (mM)/(Vol.%)		
	A	B	C
SERIES 1 - Constant HCO_3^- (respiratory changes)	16/2.5	16/5	16/10
SERIES 2 - Constant CO_2 (metabolic changes)	8/5	16/5	32/5
SERIES 3 - Constant pH (compensated changes)	8/2.5	16/5	32/10

Rat Hemidiaphragm Preparation

The rat phrenic nerve hemidiaphragm *in vitro* preparation was originally described by Bülbring (1946). This tissue was chosen for these experiments because it is a thin skeletal muscle which is easily oxygenated and supplied with nutrients. The preparation is stable over several hours if left undisturbed. It contains mostly fast muscle fibers and can be dissected out with the phrenic nerve for indirect stimulation or without the nerve for direct stimulation.

Surgery was performed on rats during ether anesthesia. The abdominal cavity was opened by a ventral incision and the intestines were pushed aside to expose the dorsal aorta. Arterial blood was drawn into a heparinized syringe and plasma was separated by centrifugation for sodium and potassium determinations, performed on an Instrumentation Laboratory 343 flame photometer, against a lithium standard. Animals were then decapitated and the skin removed from the chest. The thoracic wall was cut to expose the rostral surface of the diaphragm and the phrenic nerves. When the phrenic nerve was to be isolated with the hemidiaphragm, it was tied close to the thymus and cut, then carefully dissected away from any attaching tissues. The diaphragm was cut down the midline, and the ribs and tissues connecting each hemidiaphragm were cut away. The hemidiaphragms were then removed from the animal and placed in oxygenated Tyrode solution where the remainder of the surgery was performed. Extraneous tissues were then cut away from the hemidiaphragm leaving a section

of the ribs attached to the muscle tendons. A long piece of silk thread was tied to the central tendon and two loops of silk thread were tied to each edge of the rib segment. For direct stimulation, two teflon-coated stainless steel wire electrodes were tied to the muscle. The preparation was mounted by the two loops to a stationary acrylic hook at the bottom of a cylindrical glass bath containing 45 ml of Tyrode solution bubbled with the appropriate gas mixture at 37° C. The silk thread attached to the central tendon was tied to a Myograph-B transducer at one gram of resting tension. For indirect stimulation experiments, the phrenic nerve was passed through two silver ring electrodes imbedded in insulating dental acrylic. Muscles were stimulated with slightly more than enough voltage to elicit a maximum contractile response. The muscles were stimulated at five second intervals, with square wave monophasic pulses, for 5 milliseconds duration for direct stimulation or for 0.5 milliseconds for indirect stimulation. Contractions were recorded on a Narco Bio-Systems Physiograph equipped with a low-pass filter set at 10 cycles per second.

Contraction Experiments

Experiments were performed on paired hemidiaphragms from the same rat in the same series of buffers (see Table 1). In contraction experiments, both hemidiaphragms were first equilibrated and stimulated in Tyrode solution containing 16 mM

bicarbonate bubbled with 95% oxygen, and 5% carbon dioxide. During this stabilization period the buffer was changed every twenty minutes. The stabilization period lasted until a stable base line and a stable peak contraction height were obtained, usually about one hour. Subsequently, one of the paired hemidiaphragms was bathed and stimulated first in buffer A, then buffer B, then buffer C, then again in buffer A, while the companion hemidiaphragm was bathed and stimulated first in C, then in B, then in A, then again in C (see Table 1). Muscles remained in each buffer for 10 minutes, then the bath was drained and fresh buffer of the same type was added for 20 minutes. Thus the contraction experiments lasted 2 hours after the stabilization period. If a muscle showed signs of deterioration, that muscle was eliminated from consideration. Contractile responses on the physiograph trace were measured 15 minutes after each of the four buffer changes. The first and the last contractile responses were averaged to obtain one value for a response in that buffer. For statistical comparison, individual muscle contractions in each buffer were expressed as a percent of the maximum stable contraction recorded for that hemidiaphragm, at any time.

Diaphragms from potassium-deficient rats were studied in all three series of buffers in Tyrode solution containing 0.5 mM potassium. These muscles were stimulated directly with stainless steel electrodes. Potassium-deficient rat diaphragms were also examined in series three, (constant pH buffers, containing 0.5 mM potassium) stimulated indirectly via the phrenic nerve. Diaphragms from control rats were

studied in all three series of buffers. Hemidiaphragms were stimulated directly in these experiments which were conducted in both 5 mM potassium Tyrode solution and in 0.5 mM potassium Tyrode solution.

The effect of d-tubocurarine on directly stimulated and indirectly stimulated muscle response was examined in several pairs of hemidiaphragms. The paralyzing effect of regular insulin (Lilly) on potassium-deficient diaphragm muscle was also tested at the end of several contraction experiments.

Intracellular pH and Tissue Cation Determinations

Intracellular pH was determined on isolated rat hemidiaphragms according to the method of Adler et al. (1965). Paired hemidiaphragms were isolated, mounted, and stimulated in the manner described in the preceding section. Muscles were equilibrated for at least one hour in 16 mM bicarbonate buffer, bubbled with a 95% oxygen, 5% carbon dioxide gas mixture. After this equilibration period, buffer A of a particular series was placed in the bath containing one hemidiaphragm and buffer C of that same series was added to the bath containing the companion hemidiaphragm. Fresh buffer was supplied after 10 minutes. After 20 minutes, the buffers were switched, and contractions were recorded for 30 minutes; the buffer was renewed after 10 minutes, as usual. After 30 minutes, these same buffers containing 200 microcuries per liter of ^{3}H labeled mannitol and 50 microcuries per liter of ^{14}C labeled DMO, both obtained from New England Nuclear, were added to the appropriate organ

baths. The purity of these radiolabeled compounds was verified with paper chromatography. These buffers were renewed after 30 minutes. After 30 minutes 2 ml samples of the bath solutions were taken for analysis and the experiment was terminated. Thus each muscle was equilibrated and stimulated for one hour in buffer B, then for 1/2 hour in either buffer A or C, then for 1 1/2 hours in either C or B, so that each muscle was tested in buffers A, B, and C, one ending in buffer C and one ending buffer A. During the last hour, each buffer contained radiolabeled DMO and radiolabeled mannitol. If either hemidiaphragm showed signs of deterioration, the experiment was terminated and the results discarded.

After completion of the contraction phase of the experiment, the muscles were removed and cut away from the attaching ribs. The tendon was cut away and each muscle was cut into two parts. One piece of each muscle was placed into a tared polyethylene vial. Both vessels were weighed. The piece of muscle in the crucible was dried overnight at 100° C and reweighed, then ashed overnight at 600° C in a muffle oven. The ash was then dissolved in concentrated HCl and the calcium content of the ash was determined on a Perkin-Elmer atomic absorption spectrometer against a lanthanum standard. Distilled water, 100 times the weight of the tissue, was added to the polyethylene vial to leach out the ions and the isotopically labeled DMO and mannitol (Lipicky and Bryant, 1966). After an overnight soak, the sodium and potassium concentrations

and the ^{14}C and ^{3}H activities of the fluid was determined. Sodium and potassium determinations were performed on an Instrumentation Laboratory 343 flame photometer against a lithium standard. Radioactivity of the solutions was determined in a Beckman model LS 200 liquid scintillation counter. The buffers were analyzed for radioactivity and sodium and potassium content in a similar manner. The ^{3}H counts were corrected for ^{14}C spectral overlap according to the formula $A_t = R_1 - (E \times A_c)$ where A_t is the corrected ^{3}H counts per minute, R_1 is the uncorrected ^{3}H counts per minute over background, E is the counting efficiency of a pure ^{14}C sample on the ^{3}H channel relative to the counting efficiency of that same pure sample on the ^{14}C channel, and A_c is the counts per minute over background of the mixed sample on the ^{14}C channel. The extracellular space in ml of each muscle was estimated as the mannitol space of that muscle, determined by the formula:

$$\frac{^{3}H \text{ counts per minute of that muscle}}{^{3}H \text{ counts per minute of 1 ml of medium}}$$

The intracellular space was estimated as the tissue water weight lost in drying minus the estimated extracellular water weight. Intracellular sodium and potassium contents were determined from the sodium and potassium concentration of the solutions in which the muscles had been soaked, minus the sodium and potassium that was calculated to have been contributed by extracellular fluid. Intracellular pH was calculated from the formula:

$$pH_i = pK_a + \log\left(\frac{C_t}{C_e} \times \frac{(V_i + V_e)}{V_i} - \frac{V_e}{V_i} \times [10^{(pK - pH_e)} + 1] - 1\right)$$

where pK_a is the ionization constant of DMO (6.13), C_t is the concentration of DMO in the water of the entire tissue, C_e is the concentration of DMO in the extracellular fluid, V_e is the volume of the extracellular fluid, V_i is the volume of the intracellular fluid, and pH_e is the extracellular pH of the buffering medium (Waddell and Butler, 1959).

The mean intracellular muscle bicarbonate concentrations were calculated according to the Henderson-Hasselbalch equation, assuming an apparent pK_a value of 6.13 for carbonic acid in muscle. The solubility coefficient of carbon dioxide in intracellular fluid was taken to be 0.035 millimoles per liter per millimeter mercury pressure (Brown and Goott, 1963). The partial pressure of carbon dioxide in the buffer was determined from the measured pH and the Henderson-Hasselbalch equation, using 0.0241 millimoles per liter per millimeter of mercury pressure for the solubility coefficient of carbon dioxide in the buffer (Edsall and Wyman, 1958).

Intracellular pH and cation determinations were made on diaphragm muscle from potassium-deficient rats in all three series of buffers in 0.5 mM potassium. Similar determinations were made on muscle from control rats in series 3, constant pH buffers in 5 mM potassium. Contractile responses were measured at the end of each experiment and expressed as a percent of the maximum response recorded from that muscle.

Potential Measurements

Diaphragms were rapidly dissected out of rats as previously described. Strips of hemidiaphragm were placed in plexiglass beaker containing 16 mM bicarbonate buffer bubbled with 95% oxygen, 5% carbon dioxide. After 30 minutes, the strips were transferred to a 10 ml plexiglass chamber where they were perfused with Tyrode solution at a rate of approximately 10 ml per minute. Solutions were kept at 35° C and were bubbled with the appropriate gas mixture. The muscle strips were pinned by the adjoining tendons to a silicone-resin mat at the bottom of the chamber. Resting membrane potentials were recorded with standard microelectrode techniques, using glass micropipettes filled with 3 M potassium chloride. The electrodes had a tip resistance of 5-10 megohms. Prepulled microelectrodes with a 1 micron tip containing a glass fiber were obtained from Fredrick Haer and Company. These electrodes were filled by capillary action, and examined under a microscope. Potential signals were amplified on an Electronics for Life Science wide band electrometer equipped with a circuit for monitoring the electrode tip resistance. The signal from the electrometer was displayed on a Tektronic type 564B oscilloscope. The criteria followed for judging whether a potential was a valid resting potential were:

1. a resting potential had to be more negative than -40 millivolts
2. a resting potential had to be stable within 5 millivolts for one minute

Potential measurements were made on potassium-deficient and control diaphragm muscles bathed in buffers from series 1 and 2, containing 0.5 mM potassium. After the muscles were transferred to the chambers, the chambers were perfused with either buffer A or C of the series. After 20 minutes, 6 resting potentials were recorded. The chambers were then perfused with the other buffer of that series, and after 20 minutes, 6 potentials were again recorded.

Resting potentials of diaphragm muscles from control and from potassium-deficient rats were also determined in 16 mM bicarbonate buffer bubbled with 95% oxygen, 5% carbon dioxide, but containing different concentrations of potassium. These potassium concentrations were 20 mM, 10 mM, 5 mM, 2.5 mM, and 1.25 mM. Equilibration time in these experiments was 5 minutes, since it was reasoned that the extracellular fluid potassium will equilibrate in this time. Six potentials were recorded in each muscle at each potassium concentration.

To eliminate bias during the potential recording process, the buffers were presented to the investigator in a sequence unknown to him. The buffer sequence was revealed after completion of the experiment.

RESULTS

Contraction Studies

Action of d-tubocurarine on Directly and Indirectly Stimulated Muscle

When hemidiaphragms were stimulated directly or indirectly, a stable base line and peak contraction height were usually evident on the physiograph trace after a short equilibration period. Figure 1 shows the result of the addition of blocking doses of d-tubocurarine on paired hemidiaphragms, one stimulated directly via electrodes tied adjacent to the muscle fibers and one stimulated via the phrenic nerve. It can be seen that d-tubocurarine, at a bath concentration of 10^{-4} M, had no effect on the contractile response of the directly stimulated muscle. A lesser concentration, 10^{-6} M, almost completely blocked the contractile response of the indirectly stimulated muscle within 10 minutes. When the bath was flushed and normal Tyrode solution was again added, contractile responses to phrenic nerve stimulation began to return immediately.

Figure 1. Effect of d-tubocurarine (DTC) on directly stimulated hemidiaphragm muscle (top trace), and on indirectly stimulated muscle (bottom trace). Doses were 10^{-4} M and 10^{-6} M respectively. Marked intervals correspond to 1 minute.

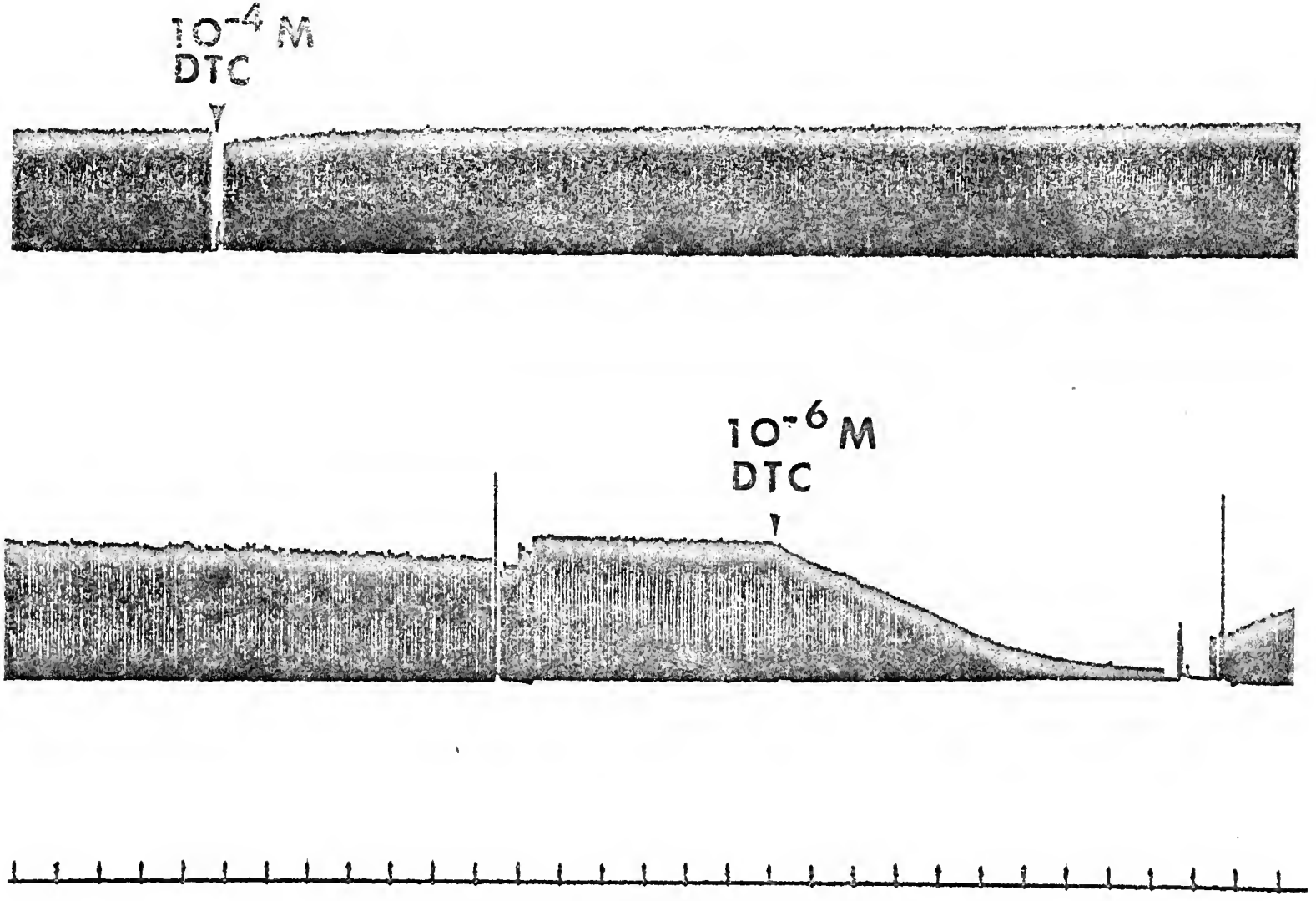

figure 1

Effect of Variations in Bicarbonate Buffer Components on Contractile Response

Rat diaphragm muscle responded to changes in bath solution pH by contracting with significantly[1] less force in more acidic buffers. This was true when acidosis resulted from an increase in the carbon dioxide concentration in the Tyrode solution (series one buffers) or from a reduction in the bicarbonate concentration in the Tyrode solution (series two buffers). Figures 2 and 3 show the contractile response of hemidiaphragm muscles from control rats plotted as a function of the pH in the Tyrode solutions. The data in Figure 2 represent muscle response in 5 mM potassium buffers, and the data in Figure 3 represent muscle response in 0.5 mM potassium buffers. It can be seen that reducing the pH of the bath fluid from 7.8 to 7.1 resulted in approximately a 20% reduction in contractile response. Figure 4 shows the effect of alterations in bath solution pH on contractile response of diaphragm muscle from potassium-deficient[2] rats. These hemidiaphragms, bathed in Tyrode solution containing 0.5 mM potassium, showed approximately a 65% reduction in

---

[1] Differences described as significant in this paragraph have a p value of less than 0.05 in the F-test.

[2] Serum potassium concentrations of rats on potassium-deficient rat chow diets averaged 1.78 mM with a standard error of 0.076 mM; n=18. When hemidiaphragms from several potassium-deficient rats were challenged with insulin, 20 units per liter, the contractile response to direct stimulation was reduced considerably; contractile response was restored when the bath potassium concentration was elevated from 0.5 mM to 5 mM, behavior characteristic of potassium-deficient rat diaphragm.

Figure 2. Contraction of control muscles in 5 mM potassium is plotted as a function of the pH of the bathing fluid. Filled circles show values obtained in experiments done in series 1, constant HCO_3^- buffers. Empty circles show values obtained in series 2, constant CO_2 buffers. The bars represent standard errors. Responses within both experiments differed significantly at $p=0.05$, in the F-test. $n=6$, for each point. For further details, see Methods, page 22.

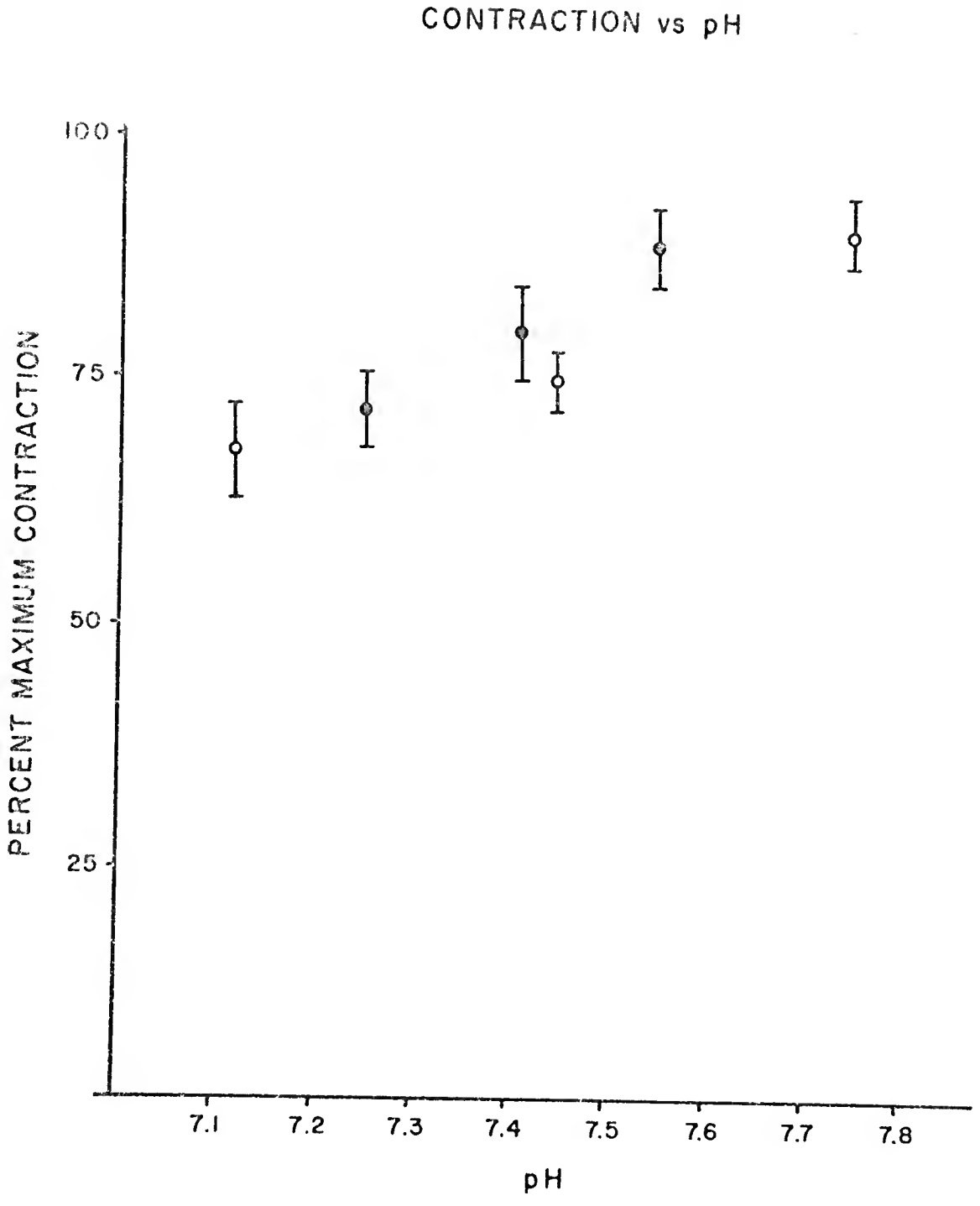
CONTRACTION vs pH
PERCENT MAXIMUM CONTRACTION
100
75
50
25
7.1
7.2
7.3
7.4
7.5
7.6
7.7
7.8
pH

FIGURE 2

Figure 3. Contraction of control muscles in 0.5 mM potassium is plotted as a function of the pH of the bathing fluid. Filled circles show the values obtained in experiments done in series 1, constant HCO_3^- buffers. Empty circles show values obtained in series 2, constant CO_2 buffers. The bars represent standard errors. Responses within both experiments differed significantly at $p=0.05$, in the F-test. n=6, for each point. For further details, see page 22.

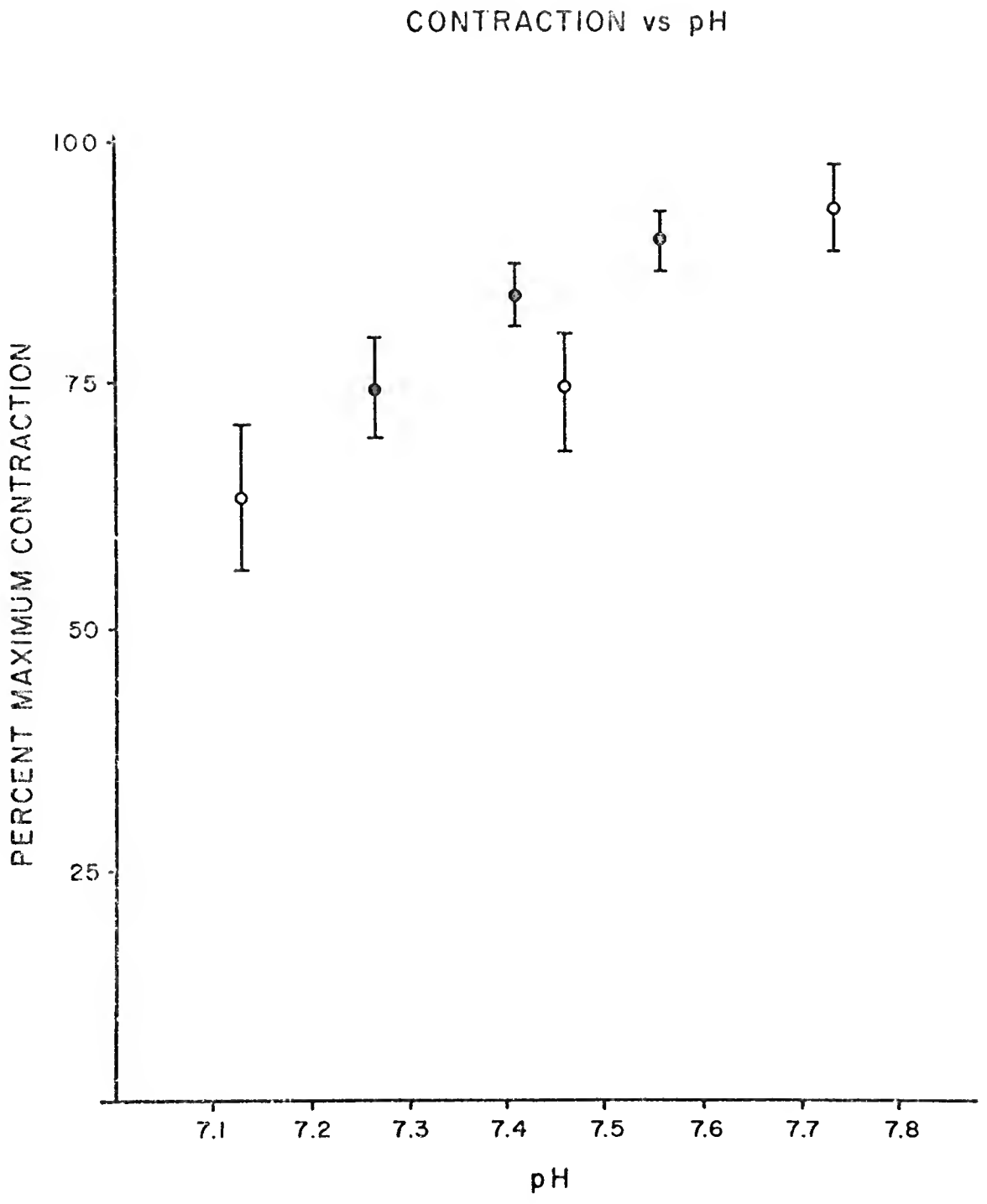
CONTRACTION vs pH
PERCENT MAXIMUM CONTRACTION
100
75
50
25
7.1
7.2
7.3
7.4
7.5
7.6
7.7
7.8
pH

FIGURE 3

Figure 4. Contraction of potassium-deficient muscles in 0.5 mM potassium is plotted as a function of the pH of the bathing fluid. Filled circles show the values obtained in experiments done in series 1, constant HCO_3^- buffers. Empty circles show values obtained in series 2, constant CO_2 buffers. The bars represent standard errors. Responses within both experiments differed significantly at $p=0.05$, in the F-test. $n=6$, for each point. For further details, see page 22.

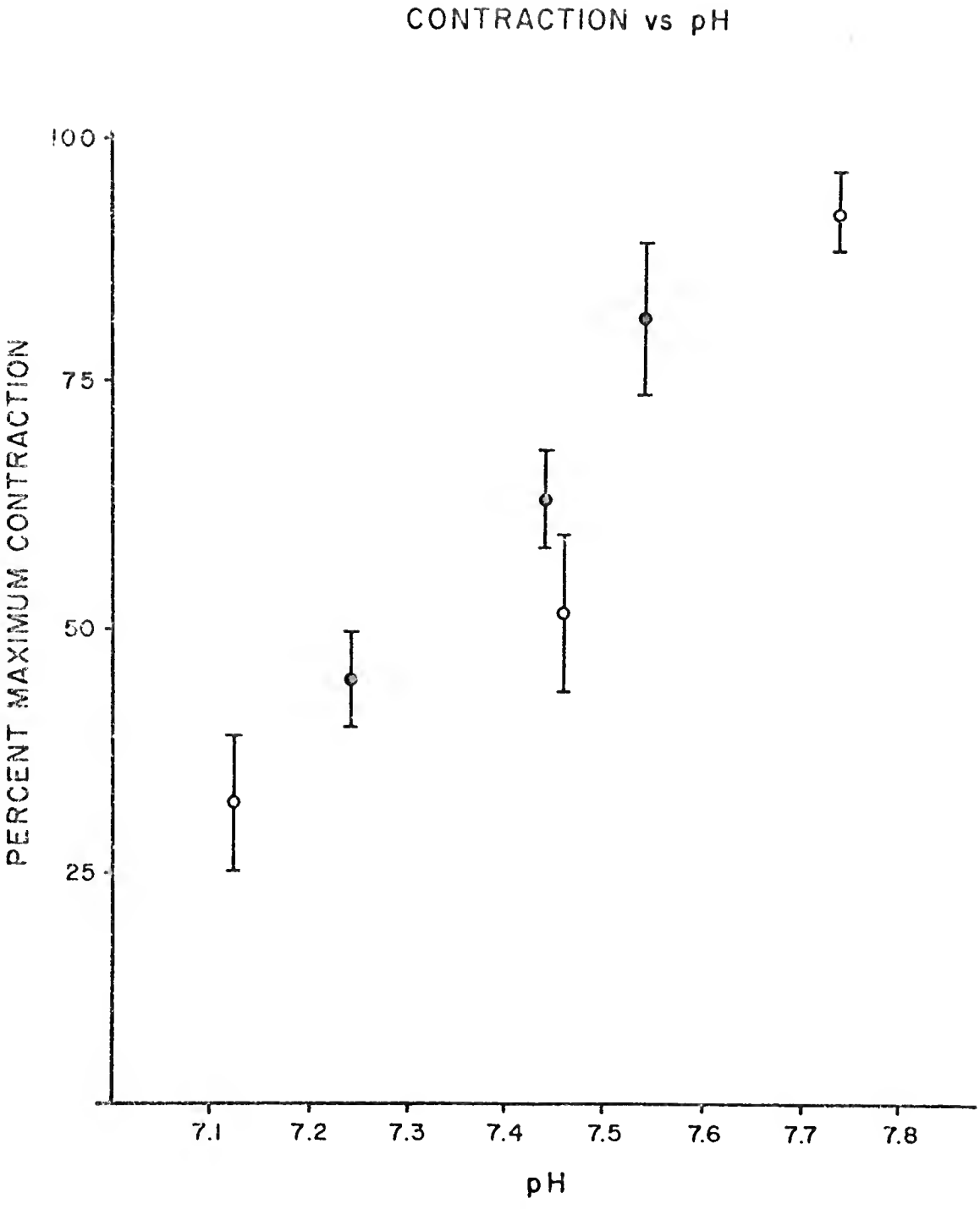
CONTRACTION vs pH
PERCENT MAXIMUM CONTRACTION
100
75
50
25
7.1
7.2
7.3
7.4
7.5
7.6
7.7
7.8
pH

FIGURE 4

Figure 5. Contraction of control muscles in 5 mM potassium is plotted as a function of the CO_2 volumes percent bubbling the buffer and the HCO_3^- concentration of the buffer. The values shown were obtained in series 3, constant pH buffers. The bars represent standard errors. Responses did not differ significantly at p=0.10, in the F-test. n=6, for each point. For further details, see page 22.

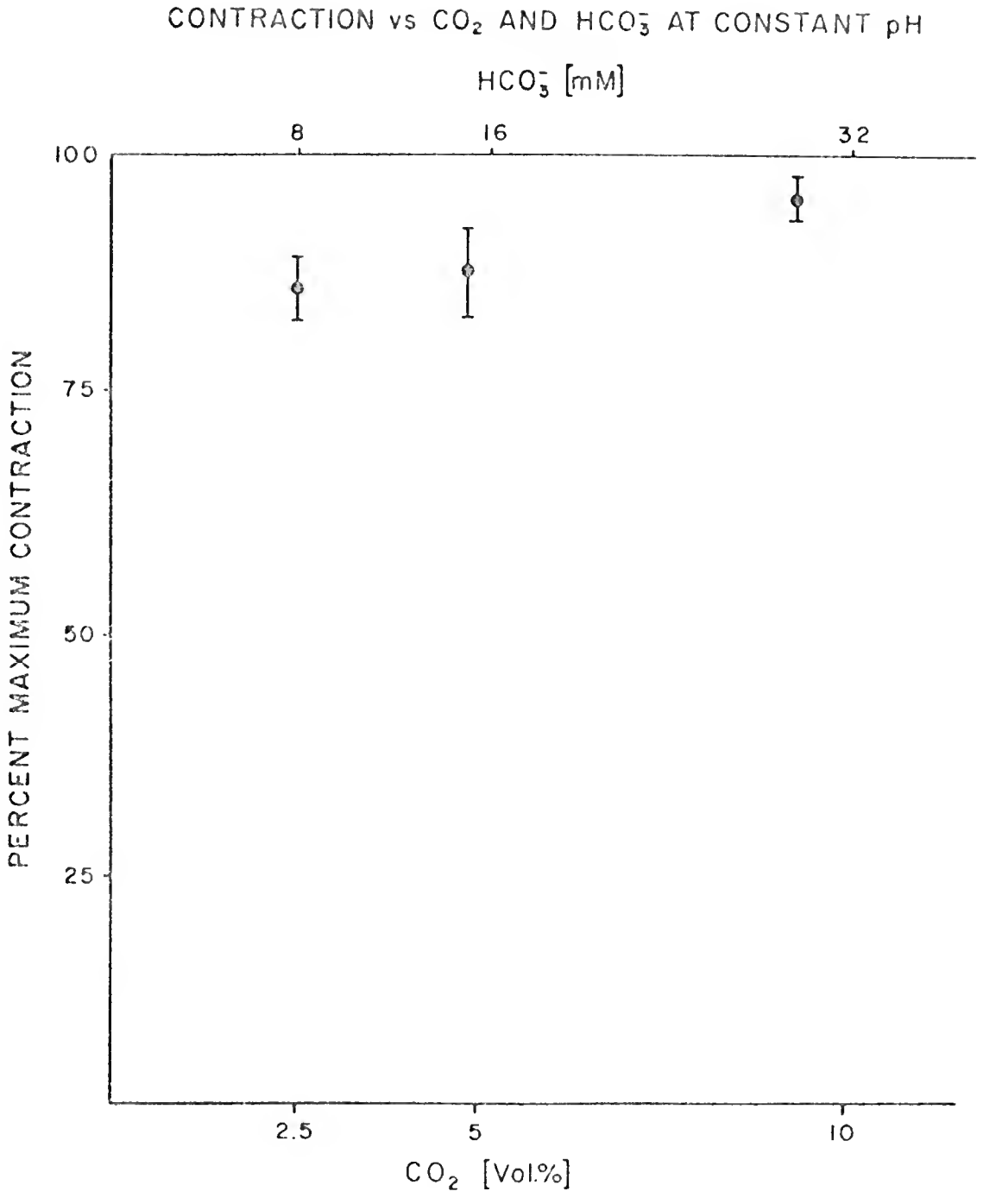
CONTRACTION vs CO_2 AND HCO_3^- AT CONSTANT pH
HCO_3^- [mM]
8
16
32
100
75
50
25
PERCENT MAXIMUM CONTRACTION
2.5
5
10
CO_2 [Vol.%]

FIGURE 5

Figure 6. Contraction of control muscles in 0.5 mM potassium is plotted as a function of the CO_2 volumes percent bubbling the buffer and the HCO_3^- concentration of the buffer. The values shown were obtained in series 3, constant pH buffers. The bars represent standard errors. Responses did not differ significantly at p=0.10, in the F-test. n=6, for each point. For further details, see page 22.

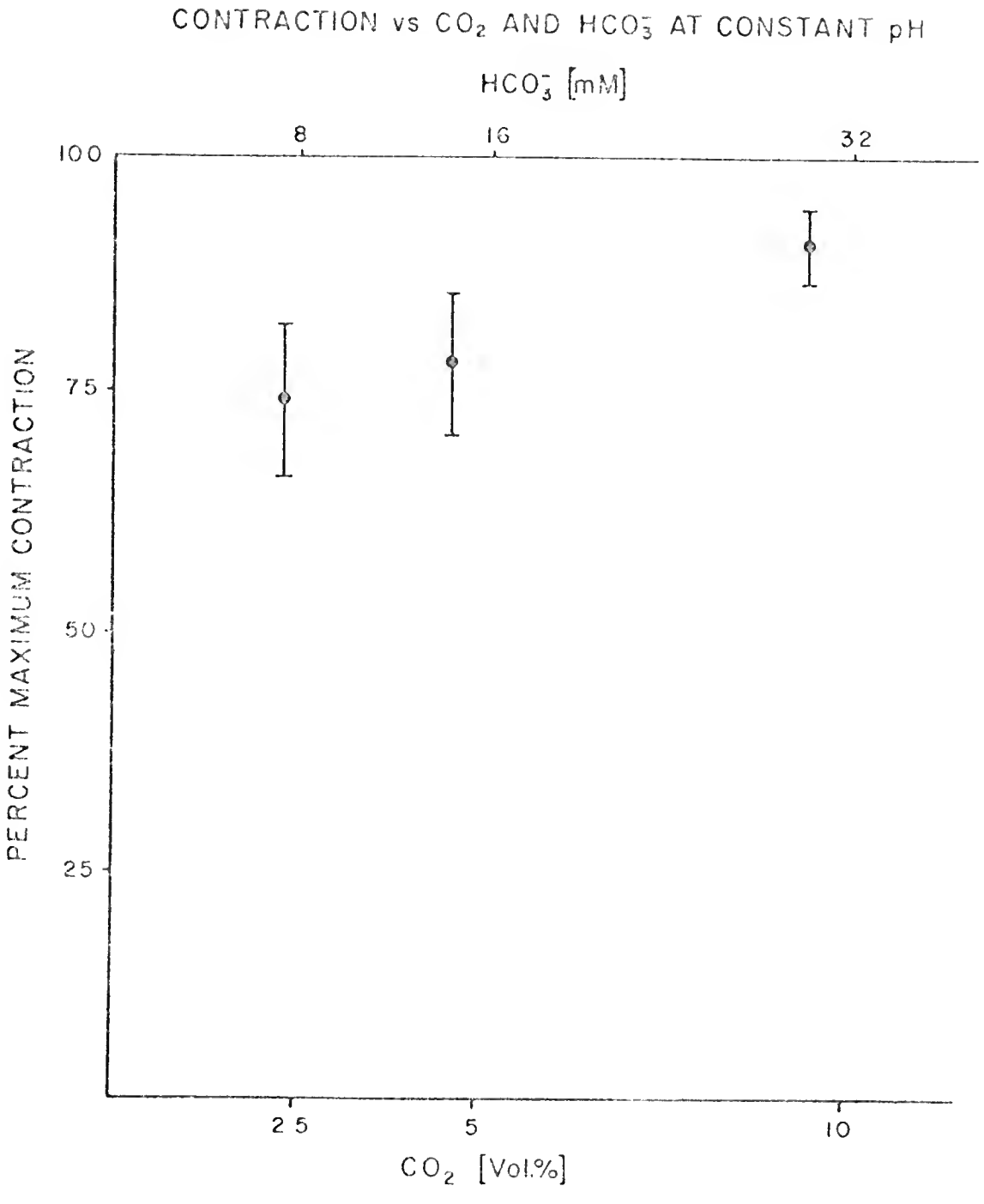
CONTRACTION vs CO_2 AND HCO_3^- AT CONSTANT pH
HCO_3^- [mM]
8
16
32
100
75
50
25
PERCENT MAXIMUM CONTRACTION
2.5
5
10
CO_2 [Vol.%]

FIGURE 6

Figure 7. Contraction of potassium-deficient muscles in 0.5 mM potassium is plotted as a function of the CO_2 volumes percent bubbling the buffer and the HCO_3^- concentration of the buffer. The values shown were obtained in series 3, constant pH buffers. Filled circles show values obtained in experiments when muscles were stimulated directly, as usual. Empty circles show values obtained when the muscles were stimulated indirectly, via the phrenic nerve. Triangles show values obtained when the muscles were stimulated directly in buffers with $SO_4^=$ and sucrose substituted for HCO_3^- in the reduced HCO_3^- solutions. Muscle performance in $SO_4^=$ and sucrose buffers was generally poor. The bars represent standard errors. Responses within all three experiments differed significantly at $p=0.05$, in the F-test. $n=6$, for each point. For further details, see page 22.

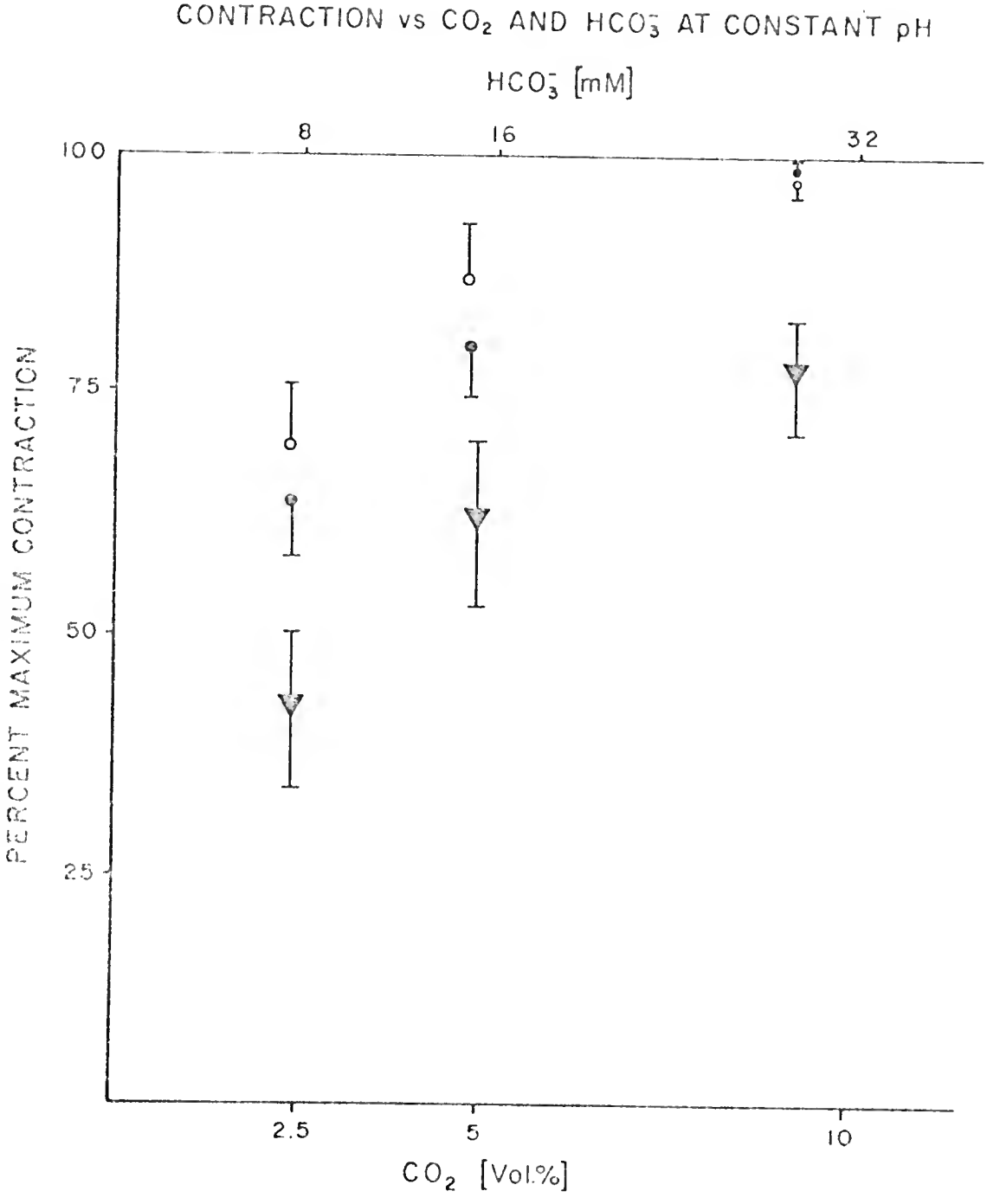
CONTRACTION vs CO_2 AND HCO_3^- AT CONSTANT pH
HCO_3^- [mM]
8
16
32
100
75
50
25
PERCENT MAXIMUM CONTRACTION
2.5
5
10
CO_2 [Vol.%]

FIGURE 7

contractile response when the pH of the bath solution was reduced from 7.8 to 7.1. This was a quantitatively greater response than that which was seen in the control rat muscles. In all of these experiments, the magnitude of the change in muscle contractility seemed independent of whether the change in pH was effected by respiratory of metabolic changes. Figures 5 through 7 show results of contraction experiments done in series three, (constant pH buffers). In these experiments, contraction of hemidiaphragm muscles was recorded in buffers containing different bicarbonate concentrations bubbled with different carbon dioxide mixtures, but with the same ratio of bicarbonate to carbon dioxide. Figures 5 and 6 show contraction of hemidiaphragms from control rats plotted as a function of both carbon dioxide volumes percent in the gas mixture bubbling the buffer solution and the bicarbonate concentration of the solution. The points in Figure 5 represent the responses recorded in muscles in Tyrode solution containing 5 mM potassium; those in Figure 6 represent responses exhibited by the muscles in 0.5 mM potassium Tyrode solution. There was no statistically significant difference in the responses exhibited by the muscles in either experiment, although there appeared to be a trend toward more vigorous contractions in buffers containing elevated bicarbonate concentrations and elevated carbon dioxide tensions. This trend reappeared in experiments done on hemidiaphragm muscles taken from potassium-deficient animals. Figure

7 shows the results of experiments which were done on hemidiaphragms from potassium-deficient rats in series three, (constant pH buffers). These buffers contained 0.5 mM potassium. In figure 7, the results of three different experiments are shown. Again, contractile response is plotted as a function of both buffer bicarbonate concentration and carbon dioxide volumes percent of the gas mixture. One set of points represents the response of muscles to direct stimulation; one set of points represents the response of muscles to indirect stimulation via the phrenic nerve; and one set of points represents the response of muscles to direct stimulation when sulfate and sucrose rather than chloride were substituted for bicarbonate in the reduced bicarbonate solutions. In all three experiments, the contractile responses were significantly greater in the high bicarbonate, high carbon dioxide buffers than in low bicarbonate, low carbon dioxide buffers.

In summary, muscle contractile force was always greater in buffers with high pH levels or high bicarbonate concentrations. Muscle contractile force increased with increasing carbon dioxide tension in constant pH experiments, but decreased with increasing carbon dioxide tension in constant bicarbonate experiments. These relationships were always more pronounced in potassium-deficient rat muscles than in muscles from control rats.

Changing the potassium concentration from 0.5 mM to 5 mM had no perceptible effect on contraction of either potassium-deficient rat muscle or control rat muscle. There was no

significant[3] difference in contractile force per gram of muscle between control rat muscles in 5 mM potassium and potassium-deficient rat muscle in 0.5 mM potassium. For 10 control and 10 potassium-deficient rat muscles, these values averaged 40.7 grams of force per gram of tissue and 33.5 grams of force per gram of tissue respectively, with a pooled standard deviation of 15.6 grams of force per gram of muscle tissue. These values were recorded in 32 mM bicarbonate buffers bubbled with a 95% oxygen, 5% carbon dioxide mixture.

Tissue Analysis

Effect of Bicarbonate and Carbon Dioxide Tension Changes on Muscle Water and Calcium Content

Changes in bicarbonate concentration and in carbon dioxide tension had no significant[4] effect on total muscle water or extracellular fluid volume. There was no significant difference between the means of these values obtained from potassium-deficient animals and the means obtained from control animals. For all experiments, 78% of muscle wet weight

[3]The difference between these two means has a p value greater than 0.05 in the Student's t-test.

[4]Differences described as significant in this section, unless otherwise noted, have a p value less than 0.05 in the paired t-test.

was water, and 36% of muscle wet weight was extracellular fluid[5]. The standard deviations of these two values were 2% and 5%, respectively.

Buffer changes had no significant effect on intracellular calcium content of muscles, nor was there any significant difference between the mean of this value determined in control rats and the mean of this value determined in potassium-deficient rats. For all tissues, the single mean and the standard deviation were 3.1 mM and 1.4 mM, respectively.

Effect of Buffer Bicarbonate and Carbon Dioxide Tension Changes on Muscle Intracellular pH and Potassium Content

A decrease in extracellular pH caused by an increase in buffer carbon dioxide tension or by a decrease in buffer bicarbonate concentration, was accompanied by a significantly lower intramuscular pH. Changes in buffer carbon dioxide resulted in greater changes in intracellular pH than changes in buffer bicarbonate. The muscles in the more acidic buffers also contained less intracellular potassium than those in

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[5]Because of the consistency of the measured tissue water weights, it was reasoned that the variation in the mannitol space measurements did not reflect true variations in the extracellular fluid volume, for any change in extracellular fluid volume would necessitate an exactly opposite change in the intracellular fluid volume. Therefore, in calculations requiring a value for the extracellular fluid volume, the average 36% of muscle wet weight was used to approximate this value.

alkaline buffers. Muscle twitch tension, measured at the end of the experiment after 1 1/2 hours total time in the last buffer, was always significantly greater in the more alkaline buffers. These results, obtained in potassium-deficient rat muscle, are shown in Tables 2 and 3.

The results of tissue analysis on potassium-deficient and control rat muscles in series 3, constant pH buffers, are shown in Tables 4 and 5. Muscles bathed in high bicarbonate, high carbon dioxide buffers were significantly more acidotic than companion muscles bathed in low bicarbonate, low carbon dioxide buffers. Muscles in the high bicarbonate, high carbon dioxide buffers also contained significantly more potassium than the hemidiaphragm muscles in low bicarbonate, low carbon dioxide buffers. Contraction, measured after 1 1/2 hours of equilibration, was always significantly greater in the high bicarbonate, high carbon dioxide buffers. Figure 8 is the physiograph trace recording of contractile responses of paired hemidiaphragm muscles from a potassium-deficient rat. The trace shows the response of the muscles before and after a change in the buffer. It can be seen that changing from a low bicarbonate, low carbon dioxide buffer to a high bicarbonate, high carbon dioxide buffer resulted in a stronger muscle response in one of the muscles while changing the buffers in the opposite direction resulted in a weaker contractile response from the companion muscle. Both buffers had a pH of 7.39.

Table 6 summarizes the pertinent data shown on Tables 2 through 5. Shown in this table are the intracellular

TABLE 2

TISSUE ANALYSIS OF HEMIDIAPHRAGMS, FROM K^+ DEFICIENT RATS, IN DIFFERENT CO_2 CONCENTRATIONS

Buffer Series 1

HCO_3^-	(Vol. %) CO_2	pH_e	pH_i[a]	$HCO_3^-{}_i$[b]	K^+_e	K^+_i (mM)[a]	% Maximum[a] Contraction
16 mM	2.5	7.64	7.36±.07 n=5	11.6 mM	0.5 mM	128±15 n=5	88±5.5 n=5
			[c]			[d]	[c,e]
16 mM	10	7.05	6.94±.03 n=5	17.2 mM	0.5 mM	108±9.1 n=5	57±5.9 n=5

[a] data presented as means ± S.E.

[b] calculated from mean pH_i

[c] significant difference at p=0.05 in paired t-test

[d] no significant difference at p=0.05 in paired t-test

[e] these values were determined at the end of the experiment and reflect a gradual waning of contractile response over the three hour experimental period

TABLE 3

TISSUE ANALYSIS OF HEMIDIAPHRAGMS, FROM K^+-DEFICIENT RATS, IN DIFFERENT HCO_3^- CONCENTRATIONS

Buffer Series 2

HCO_3^-	(Vol.%) CO_2	pH_o	pH_i[a]	$HCO_3^-{}_i$[b]	K^+_o	K^+_i (mM)[a]	% Maximum[a] Contraction
9 mM	5	7.12	6.63±.09 n=6	4.0 mM	0.5 mM	94±6.5 n=7	51±7.2 n=5
			c			c	c,d
32 mM	5	7.62	6.77±.17 n=6	6.3 mM	0.5 mM	115±4.1 n=7	70±5.4 n=5

[a] data presented as means ± S.E.

[b] calculated from mean pH_i

[c] significant difference at p=0.05 in paired t-test

[d] these values were determined at the end of the experiment and reflect a gradual waning of contractile response over the three hour experimental period

TABLE 4

TISSUE ANALYSIS OF HEMIDIAPHRAGMS, FROM K^+-DEFICIENT RATS, IN CONSTANT pH BUFFERS

Buffer Series 3

HCO_3^-	(Vol. %) CO_2	pH_o	pH_i [a]	$HCO_3^-{}_i$ [b]	K^+_o	K^+_i (mM) [a]	% Maximum [a] Contraction
8 mM	2.5	7.39	6.95±.11 n=8 [c]	4.0 mM	0.5 mM	91±9.1 n=8 [c]	39±6.1 n=8 [c,d]
32 mM	10	7.39	6.68±.15 n=8	8.7 mM	0.5 mM	115±6.4 n=8	78±3.8 n=8

[a] data presented as means ± S.E.

[b] calculated from mean pH_i

[c] significant difference at p=0.05 in paired t-test

[d] these values were determined at the end of the experiment and reflect a gradual waning of contractile response over the three hour experimental period

TABLE 5

TISSUE ANALYSIS OF HEMIDIAPHRAGMS, FROM CONTROL RATS, IN CONSTANT pH BUFFERS

Buffer Series 3

HCO_3^-	(Vol. %) CO_2	pH_o	pH_i [a]	$HCO_3^-{}_i$ [b]	K^+_o	K^+_i (mM) [a]	% Maximum [a] Contraction
8 mM	2.5	7.39	7.28±.02 n=9 [c]	8.6 mM	5 mM	145±11 n=9 [c]	70±5.2 n=9 [c,d]
32 mM	10	7.39	7.19±.03 n=9	28 mM	5 mM	170±13 n=9	81±4.5 n=9

[a] data presented as mean ± S.E.

[b] calculated from mean pH_i

[c] significant difference at p=0.05 in paired t-test

[d] these values were determined at the end of the experiment and reflect a gradual waning of contractile response over the three hour experimental period

TABLE 6

COMPARISON OF ICF ACID-BASE AND K^+ CHANGES IN HEMIDIAPHRAGMS IN VARIOUS BUFFERS

Buffer Series	(Vol. %) CO_2	$HCO_3^-{}_o$	pH_i	$H_i^+/H^+{}_o$	$K^+{}_i$	% Maximum Contraction
		K^+ Deficient				
1	2.5	16 mM	7.36	1.91	128 mM	88
	10	16 mM	6.94	1.29	108 mM	57
2	5	9 mM	6.63	3.09	94 mM	51
	5	32 mM	6.77	7.08	115 mM	70
3	2.5	8 mM	6.95	2.75	91 mM	39
	10	32 mM	6.68	5.13	115 mM	78
		Control				
1	2.5	8 mM	7.28	1.29	145 mM	70
	10	32 mM	7.19	1.58	170 mM	81

Figure 8. Effect of change in buffer of directly stimulated potassium-deficient rat hemidiaphragm muscles. Buffers contained 8 mM bicarbonate or 32 mM bicarbonate bubbled with 2.5 volumes percent or 10 volumes percent carbon dioxide with oxygen. The pH of both buffers was 7.39. Buffers contained 0.5 mM potassium. Marked time intervals correspond to 1 minute.

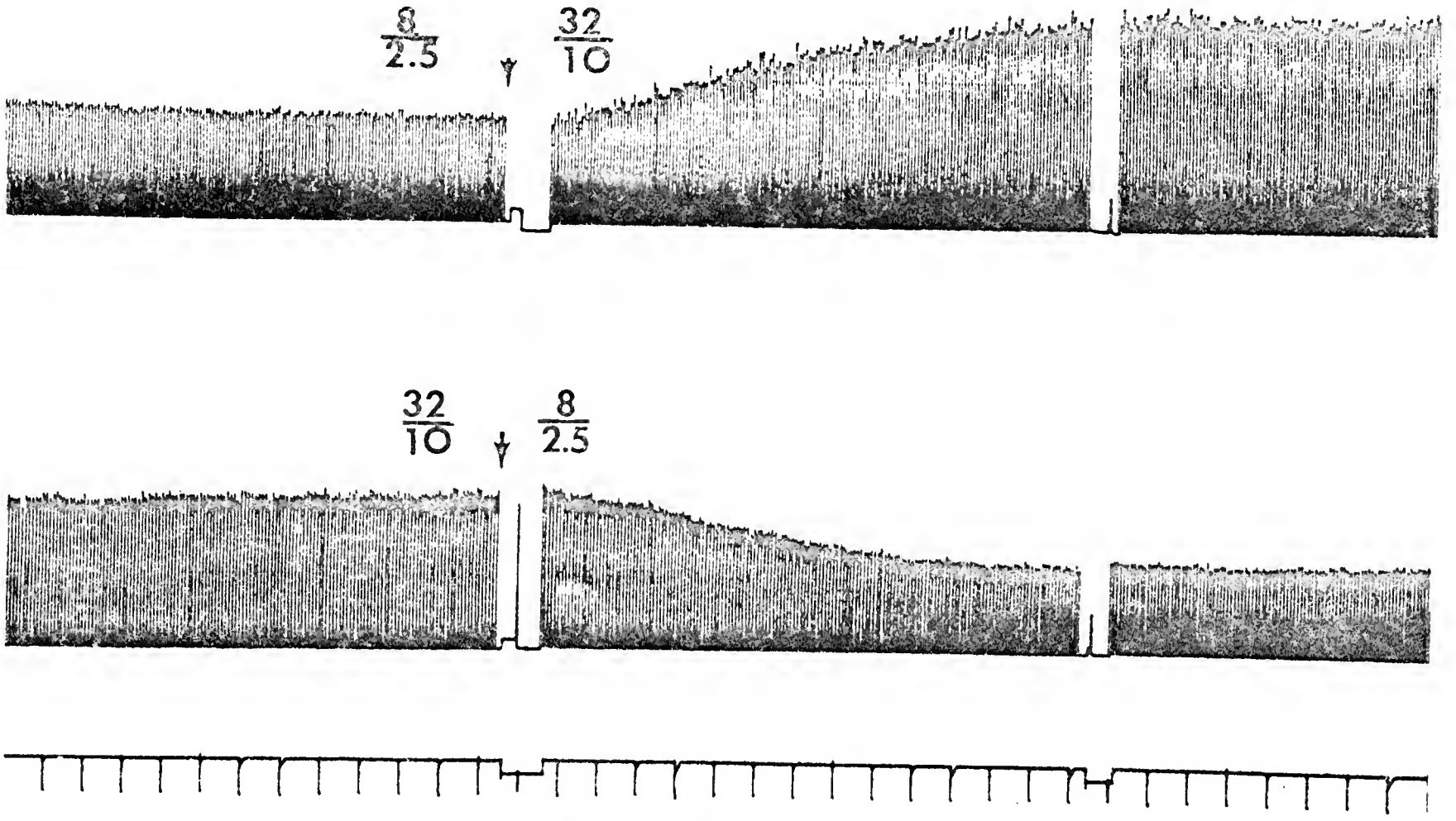

figure 8

potassium concentrations of the muscles, the contractile forces, and the ratios of intracellular to extracellular proton concentrations. It can be seen that in all four experimental pairs, the muscles which contracted with the greatest amount of force always contained the higher intracellular potassium concentration and had a higher ratio of intracellular to extracellular protons.

Resting Potential Measurements

Changing the bicarbonate concentration in the Tyrode solution from 8 mM to 32 mM had no significant effect on the resting potential of diaphragm muscles from control or from potassium-deficient rats. Changing the carbon dioxide mixture from 2.5% to 10% also had no significant effect on resting potential. These potentials were recorded in muscles bathed in 0.5 mM potassium buffers. These data are illustrated graphically in Figures 9 and 10 for potassium-deficient and control rat muscles. These graphs also show resting potentials recorded in muscles bathed in different potassium concentrations ranging from 0.5 mM to 20 mM. The recorded potentials are plotted as a function of the ratio of the intracellular potassium concentration to the extracellular (buffer) potassium concentration. The intracellular potassium concentration of potassium-deficient muscles was taken from the average of the values in Tables 2 through 4. The intracellular potassium concentration of control rat muscle was taken from the average of the values in Table 5. The horizontal bars extending from two of

Figure 9. Resting membrane potentials recorded in potassium-deficient rat hemidiaphragm muscles are plotted as a function of the ratio of intracellular to extracellular potassium concentration. Intracellular potassium concentration was estimated to be 107 mM. The horizontal bars represent the error that would be introduced if the estimated intracellular potassium concentration was incorrect by 20 mM. Vertical bars represent standard errors. The curved line is the solution to the reduced Goldman equation:

$$E_m = -61.5 \log \frac{(K^+)_i}{(K^+)_o + .01 \times (Na^+)_o}$$

The straight line is the solution to the Nernst equation,

$$E_m = -61.5 \log \frac{(K^+)_i}{(K^+)_o}$$

n=24.
The four symbols on the extreme left represent potentials recorded in buffers containing, from top to bottom, $\frac{8 \text{ mM } HCO_3^-}{5\% \ CO_2}$, $\frac{32 \text{ mM } HCO_3^-}{5\% \ CO_2}$, $\frac{16 \text{ mM } HCO_3^-}{2.5\% \ CO_2}$, and $\frac{16 \text{ mM } HCO_3^-}{10\% \ CO_2}$.
For these points only, n=12.

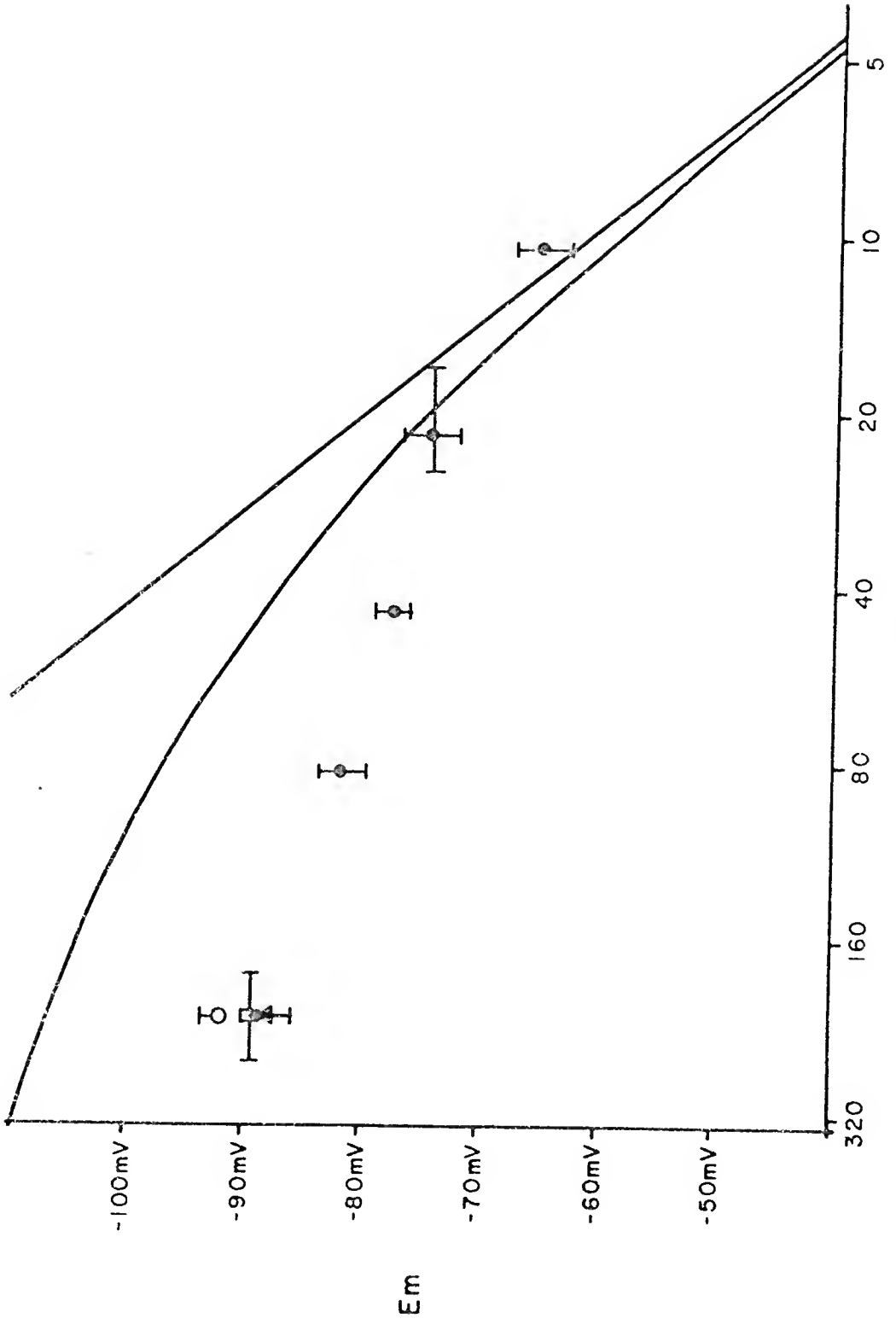

FIGURE 9

Figure 10. Resting membrane potentials recorded in control rat hemidiaphragm muscles are plotted as a function of the ratio of the intracellular to the extracellular potassium concentration. Intracellular potassium concentration was estimated to be 155 mM. Bars represent standard errors. The curved line is the solution to the reduced Goldman equation:

$$E_m = -61.5 \log \frac{(K^+)_i}{(K^+)_o + .01\ (Na^+)_o}$$

The straight line is the solution to the Nernst equation,

$$E_m = -61.5 \log \frac{(K^+)_i}{(K^+)_o}$$

n=24.
The four symbols on the extreme left represent potentials in from top to bottom, $\frac{32\ mM\ HCO_3^-}{5\%\ CO_2}$, $\frac{16\ mM\ HCO_3^-}{2.5\%\ CO_2}$, $\frac{16\ mM\ HCO_3^-}{10\%\ CO_2}$ and $\frac{8\ mM\ HCO_3^-}{5\%\ CO_2}$. For these points only, n=12.

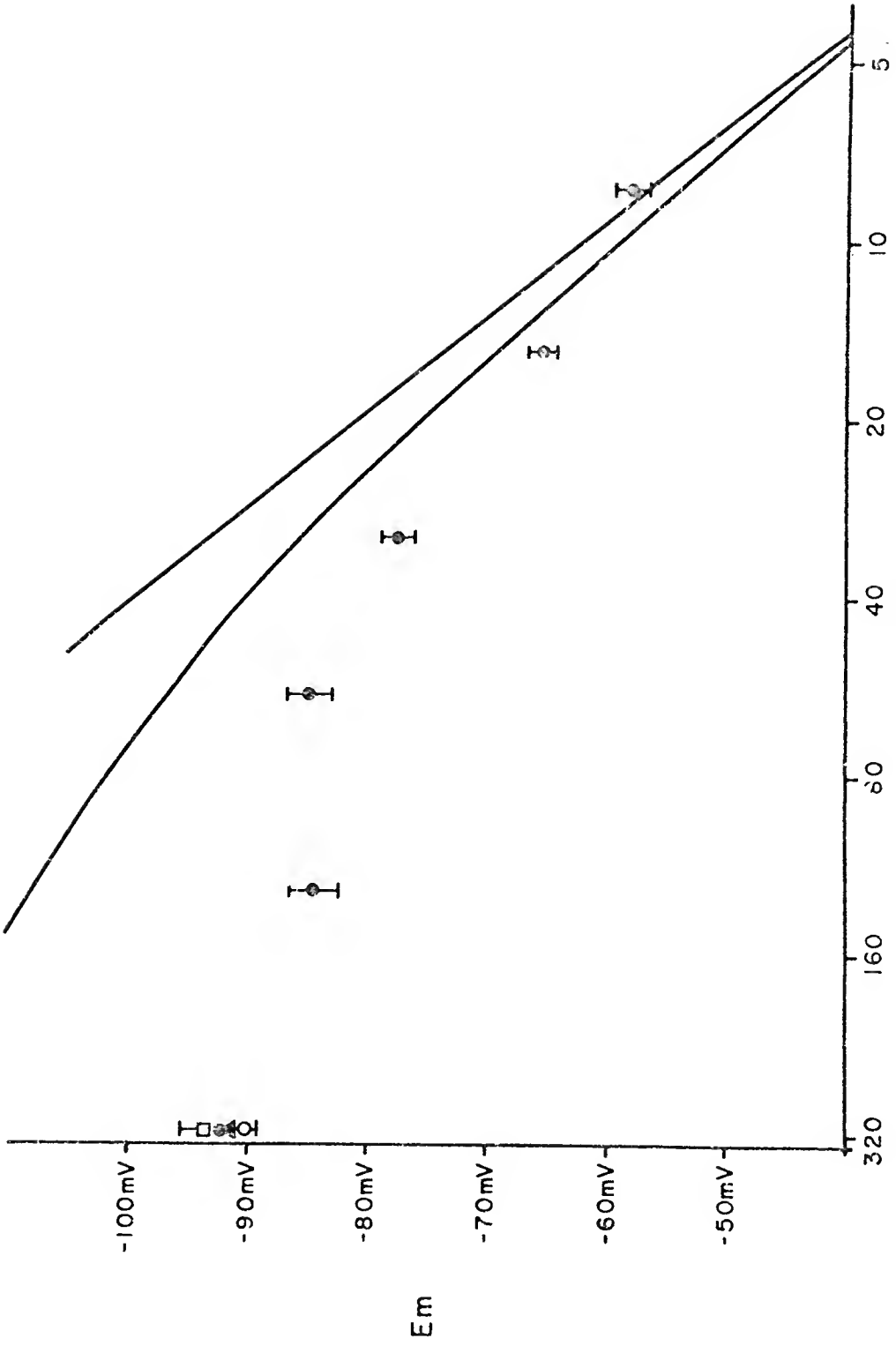

FIGURE 10

the points in Figure 9 represent the error that would be introduced if the estimated intracellular potassium concentration were incorrect by as much as 20 mM. The straight line and the smooth curve drawn on Figures 9 and 10 are, respectively, the relations described by the Nernst equation, and the reduced Goldman equation using 0.01 for the ratio of the permeability coefficients of sodium to potassium (Bilbrey et al., 1973). It can be seen that the recorded potentials do increase with decreasing extracellular potassium concentration, but not to the extent predicted by either the Nernst equation of the reduced Goldman equation. The potentials recorded in potassium-deficient rat muscles best fit the reduced Goldman equation when 0.022 was used for the ratio of the permeability of sodium to potassium. The data recorded in control rat muscles fit the equation best when 0.027 was used for the permeability coefficient ratio. All of the potential measurements shown were recorded with one of 3 microelectrodes on muscles from 2 potassium-deficient rats and 2 control rats.

DISCUSSION

The data presented in this work are the first to be reported correlating changes in muscle contraction brought about by alterations in the bicarbonate buffer system with changes in muscle ion composition. Other authors have reported that acidosis caused depression of muscle contraction, but this depression was slight in the preparations studied and the authors offered no explanation for this effect (Crul-Sluijter and Crul, 1974; Baraka, 1964). By analyzing muscles that had been contracting in different bicarbonate buffers, we hoped to determine specifically which components of the buffer system affected contraction and to elucidate the mechanism of this effect.

In the present study, acidosis mildly depressed the contractile response of control muscles. The degree of this depression was independent of the nature of the acidosis, whether caused by increased carbon dioxide tension (respiratory acidosis) or decreased external bicarbonate concentration (metabolic acidosis). These results, obtained in directly stimulated muscles, agree quantitatively with those of Crul-Sluijter (1974) who reported a 4% depression of phrenic nerve stimulated muscle contraction when HCl lowered the bath solution pH from 7.5 to 7.1. Over the same pH range, the

data in the present study show an 8% decrease in contractile response, which is within experimental error of the value reported by Crul-Sluijter.

Acidosis suppressed the contractility of muscle isolated from potassium-deficient animals to a much greater extent than muscles from control rats. These results are the first to be reported showing the increased sensitivity of potassium-depleted skeletal muscle to pH changes. A change in the buffer pH from 7.75 to 7.1 was accompanied by a 60% fall in the contractile response of this tissue compared with the 20% fall in the contactile response of muscle from control-dieted rats when pH changed over this same range. The increased sensitivity of this tissue to pH changes was not a consequence of the low potassium concentration in the buffer (0.5 mM) since control muscle contracting in 0.5 mM potassium buffer did not display any increase in sensitivity to bath pH changes, compared to control muscle in 5 mM potassium buffers.

Analysis of potassium-deficient muscles that had been contracting in buffers with different pH levels revealed that muscles in acidic buffers had lower intracellular pH values than paired muscles that had been in more alkaline buffers. The difference in intramuscular pH was greater in response to simulated respiratory changes than in response to simulated metabolic changes, although the extracellular pH changes were similar. These results are qualitatively similar to those obtained by Adler (1965) in diaphragm muscles from normal rats. Mean intracellular muscle pH values, determined in 16 mM bicarbonate solutions bubbled with

oxygen with 2.5 volumes percent carbon dioxide or 10 volumes percent carbon dioxide, both fell on the curve Adler obtained for intracellular pH values of control muscles. The two mean intracellular muscle pH values, obtained for muscles in buffers containing 8 mM bicarbonate and 32 mM bicarbonate both bubbled with 5% carbon dioxide, 95% oxygen, were lower than the two values obtained in the constant bicarbonate buffers. The results of Adler (1972) on control rat muscles depleted of potassium *in vitro* indicate that potassium-depletion tends to reduce the muscle intracellular pH. Therefore, this would predict that the intracellular pH of *in vivo* potassium-depleted muscle would be lower than the values obtained in control animals. Considering this evidence, the intracellular pH values we obtained in the constant bicarbonate experiments were high, whereas, those obtained in the constant carbon dioxide experiments were low compared to Adler's values for intracellular pH of *in vitro* potassium-depleted rat muscles. From the data in Tables 2 and 3, one can see that the muscles used in the constant carbon dioxide buffer experiments were somewhat more potassium-deficient than the muscles used in the constant bicarbonate buffer experiments. Therefore, the muscles used in the constant carbon dioxide experiments probably were also more acidotic at the beginning of the experiments. This would have contributed to the disparity between these intracellular pH values. This disparity does not detract from the essential feature which is the qualitative relationship between bicarbonate concentration and intracellular pH, and the relationship between carbon dioxide tension and intracellular pH.

Intracellular pH was found to be lower in muscles exposed to buffers with low bicarbonate concentrations compared to paired muscles in high bicarbonate buffers. Muscles in high carbon dioxide buffers were found to have a lower intracellular pH that paired muscles exposed to low carbon dioxide buffers.

Determination of muscle potassium content, performed on the same muscles used for intracellular pH determinations, showed higher intracellular potassium concentrations in muscles that had been in alkaline buffers compared to the intracellular potassium concentrations of paired muscles that had been in acidic buffers. The differences in both experiments was approximately 20 mM. Adler et al. (1965) also reported that muscles bathed in alkaline buffers had a higher intracellular potassium content than muscles bathed in acidic buffers. But the differences reported in his data were not as great as the differences found in the present study. The muscles in Adler's experiments were not contracting, while the muscles in our experiments were contracting. This difference may have been important, since Lade and Brown (1963) determined that <u>in vivo</u> decreases in muscle intracellular potassium concentration in response to acidosis took place faster when muscles were stimulated to contract. This was probably because contraction increases the rate of loss of potassium from skeletal muscle. The range of the intracellular potassium concentrations determined in potassium-deficient muscles, 90 mM to 130 mM, shows good agreement with the value of 97 mM reported by Bilbrey et al. (1973), determined on skeletal muscle from potassium-depleted rats.

Muscles exposed to high bicarbonate, high carbon dioxide buffers contracted with greater force than muscles bathed in low bicarbonate, low carbon dioxide buffers, even though these buffers had the same pH. This relationship was more pronounced in potassium-deficient muscles than in control rat muscles. This effect was independent of variations in buffer chloride concentration, and was seen in directly and indirectly stimulated muscles alike.

Tissue analysis revealed that muscles bathed in the high bicarbonate, high carbon dioxide buffers had a lower intracellular pH and a higher intracellular potassium content than muscles in low bicarbonate, low carbon dioxide buffers. Kim and Brown (1968) obtained similar results in in vivo experiments on dogs. They recorded a decrease in intracellular muscle pH when serum bicarbonate concentration and carbon dioxide tension were elevated simultaneously. They also detected an increase in extracellular potassium but did not seek the source of this potassium. In their experiments, the intracellular pH declined from 6.97 to 6.87 while the extracellular bicarbonate concentration rose from 25 mM to 65 mM, extracellular pH remained constant. The calculated change in intracellular bicarbonate was from 12 mM to 24 mM. In the present constant pH experiments with control rat muscle, intracellular pH declined from 7.28 to 7.19 while extracellular bicarbonate rose from 8 mM to 32 mM. This represents an increase in the calculated intracellular bicarbonate from 9 mM to 28 mM. In potassium-deficient rat

muscles subjected to the same treatment, intracellular pH declined from 6.95 to 6.68. The calculated intracellular bicarbonate rose from 4 mM to only 8.7 mM. These data suggest that potassium-deficient muscles are less well buffered than control muscles. Whether this means that less intracellular buffer is available for titration or that bicarbonate or proton flux is restricted in this tissue is not defined by these results.

The results of these constant pH experiments are pivotal. Comparing the results of these contraction experiments with the results of experiments conducted in non-constant pH buffers, it is clear that contractile force always increased with increasing extracellular bicarbonate concentrations and with increasing extracellular pH. However, contractile force increased with increasing carbon dioxide tension in the constant pH experiments, but decreased with increasing carbon dioxide tension in constant bicarbonate experiments. These relationships were always more pronounced in potassium-deficient rat muscle experiments than in control muscle experiments.

The paired data sets obtained from tissue analysis, summarized in Table 6, indicate that all buffer changes which effected an increase in muscle contractile force also effected an elevation in intracellular potassium. In all four experiments the difference in intracellular potassium concentration caused by the buffer change was about 20 mM. No correlation between contraction and intracellular pH was observed, however,

the ratio of the intracellular to the extracellular proton concentrations was always higher in the muscles with the higher intracellular potassium concentration. Despite the decrease in intracellular pH when extracellular pH was reduced, the ratio of intracellular to extracellular proton concentrations always fell when extracellular pH was reduced. Other authors have reported a similar relationship between potassium and hydrogen ion gradients across muscle cell membranes (Brown and Goott, 1963; Fenn and Cobb, 1934; Lade and Brown, 1963; Irvine and Dow, 1966). These results are consistent with the concept that potassium ions exchange with hydrogen ions across muscle membranes in the direction which would tend to equalize K_i^+/K_o^+ and H_i^+/H_o^+.

The redistribution of potassium brought about by the buffer changes may be responsible for the observed changes in muscle contraction. One possible mechanism whereby increased intracellular potassium concentrations might effect a change in muscle contractile force is by increasing the resting muscle membrane potential. However, the data in the present study do not support this hypothesis. Buffer changes brought about alterations in the intracellular potassium concentration of at least 20 millimoles per liter in all experiments measuring this value. As a result, the resting membrane potential would have been shifted as predicted by the Goldman equation. For potassium-deficient muscles, based on the potassium concentrations measured in Tables 2 to 4, the predicted shift in resting potential would be about 6

millivolts. In experiments recording resting muscle membrane potentials, no statistically significant shift in potential was observed in response to buffer changes of any sort. However, because our standard errors were around 7 millivolts, a shift in resting potential consistent with a 20 mM change in intramuscular potassium might not have been detected. If the average resting potential of all the muscle fibers was increased by even a few millivolts, the maximum contractile force of the muscle might be noteably increased, but the observation that changing the extracellular potassium concentration from 0.5 mM to 5 mM resulted in no change in muscle contraction argues against this theory. With a change in bath potassium of this magnitude, we measured approximately a 15 millivolt change in the resting membrane potential. Otsuka and Ohtsuki (1970) reported a 15 millivolt shift in resting rat diaphragm muscle membrane potential when the bath potassium concentration was changed from 4 mM to 0.5 mM, but saw no change in twitch tension. If a 15 millivolt shift in resting potential brought about by a change in the bath potassium concentration produced no detectable change in the contractile force, one must reject the hypothesis that a resting potential shift of 6 millivolts, effected by a change in the intracellular potassium concentration as a result of buffer alterations, could be responsible for the observed changes in muscle contractile force.

A change in the intracellular potassium concentration might affect muscle contraction by another mechanism. The

movement of calcium from the sarcoplasmic reticulum to the site which triggers muscle contraction is probably coupled to the movement of ions in the opposite direction. Potassium and magnesium are the likely candidates for this role since active transport of calcium into isolated sarcoplasmic reticulum has been shown to be coupled to magnesium and potassium ion transport out of the sarcoplasmic reticulum (Kanazawa et al., 1971). Morad and Orkand (1971) have suggested that potassium exchanges for activator calcium during contraction of cardiac muscle from frogs. If during muscle excitation intracellular potassium exchanges for calcium across the sarcoplasmic reticulum, a higher intracellular potassium concentration may facilitate this calcium movement and increase the force of the skeletal muscle contraction. This mechanism could explain why the buffer changes were more influential on contraction of potassium-deficient rat muscle. The observed changes in intracellular potassium concentration were proportionately greater in this tissue than in control muscle.

The changes in intracellular potassium concentration might be linked to changes in intracellular sodium concentration. Muscles containing less intracellular sodium might depolarize more rapidly than muscles containing more intracellular sodium. The rate of depolarization, which is in part determined by the driving force for sodium entry into cells, has been shown to influence skeletal muscle contraction (Taylor et al., 1972; Sandow, 1973). Unfortunately, it is

difficult to obtain reliable data for intracellular sodium concentrations. A better method to test this hypothesis of sodium involvement would be to record the action potentials of muscles in different buffers. Ideally, the action potentials and contractions could be recorded simultaneously. A more elaborate system could be devised to record intracellular bicarbonate activity (Khuri et al., 1974) and intracellular potassium activity as well. Experiments having this design would yield data concerning the time sequence of these events which would aid in separating cause and effect, important information that could not be obtained in the present study.

When resting membrane potentials were recorded in muscles bathed in different potassium concentrations, a smooth curve relating potential and potassium distribution was generated in which resting membrane potential continues to become more negative as extracellular potassim decreases. This is consistent with the findings of Otsuka and Ohtsuki (1970) in potassium-deficient rat diaphragm. Our recorded potential values compare favorably with the values reported by these workers. The potentials reported in the present study were not as great as the potentials the reduced Goldman equation predicts, using the value of 0.01 for the ratio of the permeabilities of sodium to potassium. Bilbrey et al., (1973) reported that resting thigh-muscle potentials, recorded *in vivo* in dogs and rats, agreed with the values predicted by this equation. The values in the present study, recorded

in vitro, fit curves described by the reduced Goldman equation with a higher value for the ratio of sodium to potassium permeability coefficients.

CONCLUSION

Elevations in buffer bicarbonate concentration or in buffer pH effected an increase in rat diaphragm muscle contractility. Elevations in carbon dioxide tension were accompanied by increases in muscle contractility when buffer bicarbonate was elevated along with and at the same ratio as carbon dioxide tension. Elevations in carbon dioxide tension brought about decreases in muscle contractility in buffers with a constant bicarbonate concentration. Hemi-diaphragm muscles from rats made potassium-deficient by diet showed a greater sensitivity to these buffer changes than muscles from control rats. Increases in muscle contractility consistently showed a positive correlation with increases in intracellular potassium concentration, but showed no consistent correlation with changes in intracellular pH. Changes in contractility and intramuscular potassium concentration did show a positive correlation with changes in the ratio of intracellular to extracellular proton concentration. Buffer changes did not effect any measurable alterations in resting muscle membrane potentials. Changes in the extracellular potassium concentration, which affected resting potentials, did not affect muscle contractility.

In contrast to results obtained in potassium-deficient and control rat muscles, contractility of skeletal muscles in patients with familial hypokalemic periodic paralysis show an unusual sensitivity to changes in extracellular potassium concentration. Gordon et al. (1970) proposed that hypokalemia renders muscle in these individuals inexcitable by causing a depolarization block. He suggested that depolarization results during hypokalemia because of a decrease in the membrane permeability for potassium. Reductions in serum bicarbonate and pH, resulting from administration of ammonium chloride or administration of acetazolamide and the subsequent renal loss of bicarbonate, prevent muscle weakness and bouts of hypokalemia (Vroom et al., 1975; Jarrell et al., 1976).

Reductions in extracellular pH and bicarbonate concentration result in improved muscle performance in hypokalemic periodic paralysis, but result in poorer muscle performance in rat muscles. However, the effect in both tissues appears to be related to changes in transmembrane potassium distribution. The results of the present study predict that both acidosis and low serum bicarbonate levels would promote a lower ratio of intracellular to extracellular potassium concentrations, but this value has never been determined in hypokalemic periodic paralysis patients treated with acetazolamide. Whether acid-base changes benefit victims of periodic paralysis by effecting a more favorable steady state potassium balance or by abolishing some other pathological change is not clear.

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BIOGRAPHICAL SKETCH

David Walter Fretthold was born on February 28, 1948, in Fairview Park, Ohio. He attended school in Rocky River, Ohio, graduating from Lutheran High School West in 1966. His education continued at Miami University in Oxford, Ohio, where he received the Bachelor of Arts and Science degree in Zoology in 1970. In 1972 he began graduate study at the University of Florida, leading to the degree of Doctor of Philosophy in the Department of Pharmacology and Therapeutics.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Lal C. Garg

Lal C. Garg, Chairperson
Associate Professor of
Pharmacology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Betty P. Vogh

Betty P. Vogh
Associate Professor of
Pharmacology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

C. Y. Chiou

C.Y. Chiou
Associate Professor of
Pharmacology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William R. Kem
Assistant Professor of
Pharmacology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Philip Posner
Assistant Professor of
Physiology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

March, 1977

Dean, College of Medicine

Dean, Graduate School